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(71) Applicant *(for all designated States except US)*: BETH ISRAEL DEACONESS MEDICAL CENTER [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: LAWLER, John, W. [US/US]; 6 Gale Road, Swampscott, MA 01907 (US).

(74) Agents: HOGLE, Doreen, M. et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

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(57) Abstract

Tumors attract blood vessels in order to grow by a process called angiogenesis. The relative quantity of stimulators and inhibitors is an important determining factor for the initiation of angiogenesis. Thrombospondins–1 and –2 are adhesive glycoproteine that have the ability to inhibit aniogenesis. This inhibiting activity has been mapped to the type 1 repeats of TSP–1 and TSP–2. The invention includes chimeric proteins that contain anti–angiogenic portions of TSP–1, TSP–2, endostatin, angiostatin, platelet factor 4, or prolactin, linked to a portion of the N–terminal region of human cartilage oligomeric matrix protein (COMP) that allows formation of pentamers. Also described herein are the nucleic acid molecules, vectors, and host cells for expressing and producing these chimeric proteins. Further embodiments of the invention include methods to treat humans or other mammals with anti–angiogenic proteins to reduce tumor size or rate of growth. Since the type 1 repeat region of TSP–1 and TSP–2 reportedly inhibits HIV infection, chimeric proteins comprising these repeats may also be used for this purpose, as well as to inhibit angiogenesis.

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COMP/TSP-1, COMP/TSP-2 AND OTHER CHIMERIC PROTEINS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/118,053 filed February 1, 1999, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Thrombospondins are a family of calcium-binding multifunctional glycoproteins that are secreted by various cell types and are developmentally regulated components of the extracellular matrix (Bornstein, P., *FASEB J., 6:*3290-3299, 1992; Bornstein, P., *J. Cell Biol., 130:*503-506, 1995). Among their functions are modulating cell attachment, migration and proliferation.

One member of this family, cartilage oligomeric matrix protein (COMP) is a pentamer in which multimerization appears to be directed by α-helical segments situated (in the amino acid sequence) either before or after the cysteine residues that form the interchain disulfide bonds. COMP has been purified (Prochownik, E.V. *et al., J. Cell Biol. 109*:843-852 (1989)). Individuals affected with pseudoachondroplasia, who have considerably shortened stature as a result of premature cessation of bone growth, have been shown to have mutations in exon 17B of the COMP protein (*Nature Genetics 10*:325-329 (1995)).

In vitro assays have shown that platelet thrombospondin-1 is involved in thrombosis, fibrinolysis, wound healing, inflammation, tumor cell metastasis and angiogenesis. The major form of thrombospondin secreted by platelets and endothelial cells is TSP-1. Thrombospondin-1 (TSP-1) is an angiogenesis inhibitor that decreases tumor growth. Thrombospondin- 2 (TSP-2) is a related glycoprotein of similar structure and properties.

The thrombospondin type 1 repeats (TSRs; also "repeat regions" herein) have been shown to inhibit angiogenesis and HIV infection. However, other portions of the proteins have been shown to have a positive effect on endothelial cell

growth. Thromobospondin-1 and -2 are similar in terms of their molecular architecture. Thrombospondin-1and thrombospondin-2 each have three copies of the TSR. TSP-1 and TSP-2 are trimeric molecules. Thus, each fully assembled protein contains nine TSRs.

Whereas TSP-1 and TSP-2 are antiangiogenic, these proteins contain other domains that have additional activities that diminish the antiangiogenic activity. The isolated TSRs are more potent inhibitors of angiogenesis than the native molecules.

The ingrowth of new capillary networks into developing tumors is essential for the progression of cancer. Thus, the development of pharmaceuticals that inhibit the process of angiogenesis is an important therapeutic goal. As pointed out in a review by Folkman (Folkman, J., *Proc. Natl. Acad. Sci. USA 95*: 9064-9066, 1998), antiangiogenic therapy has little toxicity, does not require the therapeutic agent to enter tumor cells or cross the blood-brain barrier, controls tumor growth independently of growth of tumor cell heterogeneity, and does not induce drug resistance.

SUMMARY OF THE INVENTION

The invention includes chimeric proteins comprising: (1) a chimeric protein comprising the second and third type 1 repeats of human TSP-1, and which may also comprise the procollagen homology region of TSP-1; (2) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1; (3) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1; (4) a chimeric protein comprising the multimerization domain of human COMP, the procollagen region, and the first, second, and third type 1 repeats of human TSP-1; (5) a chimeric protein comprising the three type 1 repeats of human TSP-2, and which may also comprise the procollagen homology region of TSP-2; (6) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP,

and the three type 1 repeats of human TSP-2; and (7) variants of any of the above having anti-angiogenic activity. The invention further includes isolated nucleic acids encoding any of the above chimeric proteins, vectors comprising these nucleic acids, and host cells comprising any of said vectors. The chimeric proteins can be produced in host cells and used in methods for the treatment of a disease or medical condition characterized by abnormal or undesirable proliferation of blood vessels, such as that occurring in tumor growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of the amino acid sequence of human TSP-1 (SEQ ID NO: 1). The type 1 repeats of TSP-1 are, as illustrated here, 1) amino acids 361-416; 2) amino acids 417-473; and 3) amino acids 474-530.

Figure 2 is a representation of the amino acid sequence of human TSP-2 (SEQ ID NO: 2). The type 1 repeats of TSP-2 are, as illustrated here, 1) amino acids 381-436; 2) amino acids 437-493; and 3) amino acids 494-550.

Figure 3 is a representation of the amino acid sequence of human COMP (SEQ ID NO: 3). The type 2 repeats of COMP are, as illustrated here, 1) amino acids 89-128; 2) amino acids 129-181; 3) amino acids 182-226; and 4) amino acids 227-268

Figures 4A and 4B together are a representation of the DNA sequence (SEQ ID NO: 4) of gene encoding a human COMP/TSP-1 chimeric protein and the amino acid sequence (SEQ ID NO: 5) of a human COMP/TSP-1 chimeric protein encoded by the DNA sequence above it.

Figure 5A and 5B together are a representation of the DNA sequence (SEQ ID NO: 6) of a gene encoding a human COMP/TSP-2 chimeric protein and the amino acid sequence (SEQ ID NO: 7) of a human COMP/TSP-2 chimeric protein encoded by the DNA sequence above it.

Figure 6 is a schematic representation of a few of the chimeric protein embodiments of the invention.

Figure 7 is a graph showing tumor volume (mm^3) at 7, 14 and 21 days in the experiment described in Example 3, in which mice were injected with an unaltered

(control) vector, pNeo (filled diamonds) or with an expression vector encoding COMP/TSP-1 chimeric protein (filled squares).

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a protein that has the functional activity of the TSR but not other activities associated with TSP-1 or TSP-2, and is assembled into a multimeric structure. One embodiment of the invention is a chimeric protein that comprises the TSRs from TSP-1 or TSP-2 and the multimer assembly region of human cartilage oligomeric matrix protein (COMP), using a portion of the amino-terminal end. Other portions of TSP-1 or TSP-2 can be incorporated into the chimeric protein, such as the procollagen homology region of TSP-1 and/or TSP-2. The last two TSRs of TSP-1 are preferably used because the first TSR has the ability to activate transforming growth factor β (TGF-β), which stimulates tumor growth. The COMP assembly domain spontaneously forms a 5-stranded α-helical domain, allowing for the use of the COMP domain as a tool for pentamerization.

Thus, the COMP/TSP-1 construct contains the region for multimerization, the first type 2 repeat of human COMP (construct encodes amino acids 1-128) and the second and third TSRs of human TSP-1 (construct encodes amino acids 417-530). See the Table for active sequences of TSP-1 (taken from chapter 2, "The Primary Structure of the Thrombospondins" In *The Thrombospondin Gene Family* (J.C. Adams *et al.*, eds.) Springer-Verlag, Heidelberg (1995)). The assembled protein is a pentamer containing 10 copies of the TSR. Thus, COMP/TSP-1 and COMP/TSP-2 are expected to be more active than TSP-1 and TSP-2. COMP/TSP-1 and COMP/TSP-2 are expected to be correctly folded and multimeric so that they better mimic the natural proteins than peptides that are based on the TSR sequence.

The first type 2 repeat of COMP includes amino acid residues 73-130, based on the genomic sequence. The amount of COMP sequence at the 3' end can be increased or decreased to maximize activity. For example, two or more type 2 repeats of COMP can be included if moving the type 1 repeats of TSP-1 or TSP-2 farther out on the arms of the expressed protein increases its activity. Alternatively, "spacer" sequence not naturally occurring in COMP or in TSP-1 or TSP-2 can be

added. The COMP/TSP-2 construct contains the same region of COMP and the three TSRs of human TSP-2 (construct encodes amino acids 381-550). When it is assembled to a pentamer this chimeric protein will contain 15 TSRs. Because these proteins are derived from portions of human proteins, they should not be immunogenic in humans.

Table: Active Regions of Interest Within Thrombospondin-1

Domain	Sequence	Function
Procollagen homology	NGVQYRN (SEQ ID NO: 8)	Anti-angiogenesis
Type 1 repeats	CSVTCG (SEQ ID NO: 9)	Cell binding
	WSXWSXW (SEQ ID NO: 10)	Heparin binding
	GGWSHW (SEQ ID NO: 11)	TGF-β and Fibronectin binding
	RFK	TGF-β activation
	SPWDICSVTCGGGVQKRSR (SEQ ID NO: 12)	Anti-angiogenesis
Type 2 repeats	DVDEC$(X)_6$C$(X)_8$CENTDPGYNCLPC (SEQ ID NO: 13)	Calcium binding

In one aspect, the invention comprises polynucleotides or nucleic acid molecules that encode chimeric proteins having portions whose amino acid sequences are derived from human TSP-1. By the genomic structure, the type 1 repeats of TSP-1 are amino acid residues 359-414 (first), amino acid residues 415-473 (second), and 474-531 (third). In one case, the chimeric protein encoded by the polynucleotides of the invention comprises the second and third type 1 repeats of human TSP-1. Such a chimeric protein may also comprise the procollagen homology region and the first type 1 repeat of TSP-1. If amino acid sequences that activate TGF-β are included in the product protein, and are found to reduce anti-angiogenic activity, the RFK sequence can be mutated (to QFK, for example) to a

sequence that does not activate TGF-β, by appropriate manipulations of the nucleic acid molecule or construct encoding the chimeric proteins. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity (for example, plus or minus one order of magnitude in an assay) to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 4A and 4B. In another case, the chimeric proteins encoded by polynucleotides of the invention comprise the second and third type 1 repeats of human TSP-1, the multimerization domain of human COMP, and the first type 2 repeat of human COMP. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 4A and 4B.

In one aspect, the invention comprises polynucleotides or nucleic acid molecules that encode chimeric proteins having portions whose amino acid sequences are derived from human TSP-2. The genomic structure of the human TSP-2 gene, which would provide one way to define the boundaries of the repeats, has not been determined. In one case, the chimeric protein encoded by the polynucleotides of the invention comprises the three type 1 repeats of human TSP-2. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric proteins which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 5A and 5B. In another case, the chimeric protein encoded by polynucleotides of the invention comprises the three type 1 repeats of human TSP-2, and the multimerization domain of human COMP. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 5A and 5B.

The polynucleotides of the invention can be made by recombinant methods, can be made synthetically, can be replicated by enzymes in *in vitro* (e.g., PCR) or *in vivo* systems (e.g., by suitable host cells, when inserted into a vector appropriate for replication within the host cells), or can be made by a combination of methods. The polynucleotides of the invention can include DNA and its RNA counterpart.

As used herein, "nucleic acid," "nucleic acid molecule," "oligonucleotide" and "polynucleotide" include DNA and RNA and chemical derivatives thereof, including phosphorothioate derivatives and RNA and DNA molecules having a radioactive isotope or a chemical adduct such as a fluorophore, chromophore or biotin (which can be referred to as a "label"). The RNA counterpart of a DNA is a polymer of ribonucleotide units, wherein the nucleotide sequence can be depicted as having the base U (uracil) at sites within a molecule where DNA has the base T (thymidine).

Isolated nucleic acid molecules or polynucleotides can be purified from a natural source or can be made recombinantly. Polynucleotides referred to herein as "isolated" are polynucleotides purified to a state beyond that in which they exist in cells. They include polynucleotides obtained by methods described herein, similar methods or other suitable methods, and also include essentially pure polynucleotides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant polynucleotides that have been isolated. The term "isolated" as used herein for nucleic acid molecules, indicates that the molecule in question exists in a physical milieu distinct from that in which it occurs in nature. For example, an isolated polynucleotide may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs, and may even be purified essentially to homogeneity, for example as determined by agarose or polyacrylamide gel electorphoresis or by A_{260}/A_{280} measurements, but may also have further cofactors or molecular stabilizers (for instance, buffers or salts) added.

The invention further comprises the polypeptides encoded by the isolated nucleic acid molecules of the invention. Thus, for example, the invention relates to fusion proteins, comprising a portion of TSP-1 which comprises the second and third type 1 repeats, linked to a second moiety not occurring in TSP-1 as found in

nature. In an analogous manner, the invention relates also to fusion proteins, comprising TSP-2 or a functional portion thereof such as one or more repeat regions as a first moiety, linked to second moiety not occurring in TSP-2 as found in nature. The second moiety can be an amino acid, peptide or polypeptide, and can have enzymatic or binding activity of its own. The first moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises the portion of human TSP-1 described immediately above, or human TSP-2 or a portion thereof as the first moiety, and a second moiety comprising a linker sequence and an affinity ligand.

Another aspect of the invention relates to a method of producing a chimeric protein of the invention, or a variant thereof, and to expression systems and host cells containing a vector appropriate for expression of a chimeric protein of the invention. Variants of the chimeric protein include those having amino acid sequences that differ from those sequences in Figures 4A and 4B, and Figures 5A and 5B, wherein those variants have several, such as 5 to 10, 1 to 5, or 3, 2 or 1 amino acids substituted, deleted, or added, in any combination, compared to the sequences in Figures 4A and 4B and Figures 5A and 5B. In one embodiment, variants have silent substitutions, additions and deletions that do not alter the properties and activities of the chimeric protein. Variants can also be modified polypeptides in which one or more amino acid residues are modified, and mutants comprising one or more modified residues.

Proteins and polypeptides described herein can be assessed for their angiogenic activity by using an assay such as those described in Tolsma, S.S. *et al., J. Cell Biol. 122(2)*:497-511 (1993), one which measures the migration of bovine adrenal capillary endothelial cells in culture, and one which tests migration of cells into a sponge containing an agent to be tested for activity. A further test for angiogenesis, which can also be adapted also to test anti-angiogenesis activity, is described in Polverini, P.J. *et al., Methods. Enzymol. 198*:440-450 (1991).

Cells that express such a chimeric protein or a variant thereof can be made and maintained in culture, under conditions suitable for expression, to produce protein for isolation. These cells can be procaryotic or eucaryotic. Examples of

procaryotic cells that can be used for expression (as "host cells"; "cell" including herein cells of tissues, cell cultures, cell strains and cell lines) include *Escherichia coli, Bacillus subtilis* and other bacteria. Examples of eucaryotic cells that can be used for expression include yeasts such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris* and other lower eucaryotic cells, and cells of higher eucaryotes such as those from insects and mammals. Suitable cells of mammalian origin include primary cells, and cell lines such as CHO, HeLa, 3T3, BHK, COS, 293, and Jurkat cells. Suitable cells of insect origin include primary cells, and cell lines such as SF9 and High five cells. (See, e.g., Ausubel, F.M. *et al.*, eds. *Current Protocols in Molecular Biology,* Greene Publishing Associates and John Wiley & Sons Inc., (containing Supplements up through 1998)).

In one embodiment, host cells that produce a recombinant chimeric protein, variant, or portions thereof can be made as follows. A gene encoding a chimeric protein described herein can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, virus or other suitable replicon (including vectors suitable for use in gene therapy, such as those derived from adenovirus or others; see, for example Xu, M. *et al., Molecular Genetics and Metabolism 63*:103-109, 1998) can be present in a single copy or multiple copies, or the gene can be integrated in a host cell chromosome. A suitable replicon or integrated gene can contain all or part of the coding sequence for the protein or variant, operably linked to one or more expression control regions whereby the coding sequence is under the control of transcription signals and linked to appropriate translation signals to permit translation. The vector can be introduced into cells by a method appropriate to the type of host cells (e.g., transformation, electroporation, infection). For expression from the gene, the host cells can be maintained under appropriate conditions (e.g., in the presence of inducer, normal growth conditions, etc.). Proteins or polypeptides thus produced can be recovered (e.g., from the cells, the periplasmic space, culture medium) using suitable techniques.

The invention also relates to isolated proteins or polypeptides encoded by nucleic acids of the present invention. Isolated proteins can be purified from a natural source or can be made recombinantly. Proteins or polypeptides referred to

herein as "isolated" are proteins or polypeptides purified to a state beyond that in which they exist in cells and include proteins or polypeptides obtained by methods described herein, similar methods or other suitable methods, and also include essentially pure proteins or polypeptides, proteins or polypeptides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Thus, the term "isolated" as used herein, indicates that the polypeptide in question exists in a physical milieu distinct from the cell in which its biosynthesis occurs. For example, an isolated COMP/TSP-1 or COMP/TSP-2 chimeric protein may be purified essentially to homogeneity, for example as determined by PAGE or column chromatography (for example, HPLC), but may also have further cofactors or molecular stabilizers added to the purified protein to enhance activity. In one embodiment, proteins or polypeptides are isolated to a state at least about 75% pure; more preferably at least about 85% pure, and still more preferably at least about 95% pure, as determined by Coomassie blue staining of proteins on SDS-polyacrylamide gels.

Chimeric or fusion proteins can be produced by a variety of methods. For example, a chimeric protein can be produced by the insertion of a TSP gene or portion thereof into a suitable expression vector, such as Bluescript SK +/- (Stratagene), pGEX-4T-2 (Pharmacia), pET-15b, pET-20b(+) or pET-24(+) (Novagen). The resulting construct can be introduced into a suitable host cell for expression. Upon expression, chimeric protein can be purified from a cell lysate by means of a suitable affinity matrix (see e.g., *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al*., eds., Vol. 2, pp. 16.4.1-16.7.8, containing supplements up through Supplement 44, 1998).

Polypeptides of the invention can be recovered and purified from cell cultures by well-known methods. The recombinant protein can be purified by ammonium sulfate precipitation, heparin-Sepharose affinity chromatography, gel filtration chromatography and/or sucrose gradient ultracentrifugation using standard techniques. Further methods that can be used for purification of the polypeptide include ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction

chromatography, affinity chromatography, hydroxylapatite chromatography and high performance liquid chromatography. Known methods for refolding protein can be used to regenerate active conformation if the polypeptide is denatured during isolation or purification.

The method to construct genes encoding COMP/TSP-1 or COMP/TSP-2 hybrid proteins can be applied more broadly to produce polynucleotides, and vectors and host cells comprising such polynucleotides, wherein the polynucleotides encode COMP/endostatin, COMP/angiostatin, COMP/platelet factor 4, or COMP/prolactin, for example. In each case, a portion of a polynucleotide known to encode full-length human endostatin, angiostatin, platelet factor 4 (GenBank Accession No. M25897) or prolactin (GenBank Accession No. V00566), can be chosen for cloning into a COMP cDNA as illustrated herein for COMP/TSP-1 and COMP/TSP-2 DNA constructs. Thus, the invention also includes COMP/endostatin, COMP/angiostatin, COMP/platelet factor 4, and COMP/prolactin chimeric proteins encoded by such nucleic acid constructs. See Figure 6 for a schematic representation of the structure of COMP/endostatin.

In addition, a portion of the endostatin, angiostatin, platelet factor 4 or prolactin coding regions, wherein that portion encodes a polypeptide having anti-angiogenic activity, can be added to or incorporated into a DNA construct encoding COMP/TSP-1, such that a TSP-1-derived polypeptide and a polypeptide derived from endostatin, angiostatin, platelet factor 4 or prolactin are produced fused together in tandem on the same "arm" of the "5-armed" COMP-multimerized pentamer. Different expression constructs can be introduced into the same host cells such that two or more chimeric protein "arms" of different types (e.g., COMP/angiostatin and COMP/TSP-1 or COMP/TSP-2) are joined at the COMP multimerization domain.

Chimeric protein antiangiogenic agents can be used, for example, after surgery or radiation to prevent recurrence of metastases, in combination with conventional chemotherapy, immunotherapy, or various types of gene therapy not necessarily directed against angiogenesis.

Construction of COMP/TSP-1P Expression Vectors

Expression vectors that can be used to produce COMP/TSP-1P, a chimeric protein that includes the procollagen homology region (see Figure 6), can be produced from two distinct cDNAs. The COMP portion is identical to that in the Examples described herein. For TSP-1, a new forward primer (GAT GAC GTC ACT GAA GAG AAC AAA GAG) (SEQ ID NO: 14) and the same reverse primer as described in the Examples can be used to produce a PCR product that is approximately 750 base pairs in size and has an AatII restriction endonuclease site at the 5' end and an XbaI restriction endonuclease site at the 3' end. The product codes for amino acids 284-530 and includes the procollagen homology region (exons 6 and 7) and type 1 repeats. If inclusion of the TGF-β activating sequence (RFK) that is in the first type 1 repeat is found to reduce the antitumor activity, this sequence will be mutated to an inactive sequence (QFK, for example) using an oligonucleotide-directed mutagenesis kit (Amersham). The COMP/TSP-1P expression vector can be constructed by cutting the PCR product with AatII and XbaI and cloning it into the COMP cDNA cut with the same enzymes. The protein can be expressed using the methods that have been described for COMP/TSP-1 and COMP/TSP-2.

Construction of COMP/Endostatin Expression Vectors

The strategy for making multimers of the TSP-1 and TSP-2 can be used to make multimers of other anti-angiogenic proteins. For example, if the active region of endostatin is prepared by PCR and cloned into the COMP cDNA, a pentameric structure of endostatin can be made when this construct is expressed (O'Reilly M.D., *et al.*, *Cell 88*:277-285, (1997)). In addition, if the COMP/TSP-1 and the COMP/endostatin genes are expressed concurrently within the same cells, mixed pentamers of COMP/TSP-1 and COMP/endostatin subunits are made. The mixed multimer allows simultaneous treatment with the two reagents by delivery of a single therapeutic. An additive or synergistic effect of the two agents may significantly increase the efficacy of this reagent as compared to that of each reagent alone. For example, combination therapy with angiostatin and endostatin has eradicated tumors in mice (Boehm, T. *et al., Nature 390:*404-407, 1997).

The cDNA for endostatin can be prepared by PCR of liver cDNA or from an isolated cDNA clone for collagen XVIII (GenBank accession no. L22548). The human endostatin cDNA can be produced by PCR with the forward primer GAT GAC GTC CAC AGC CAC CGC G (SEQ ID NO: 15) and the reverse primer GAT TCT AGA CTA CTT GGA GGC AGT CAT G (SEQ ID NO: 16). The resulting PCR product is approximately 560 base pairs and encodes amino acids 1 to 184 of human endostatin (Sasaki, T., *et al., EMBO J.*, *17*:4249-4256, 1998). The COMP/endostatin expression vector can be constructed by cutting the PCR product with AatII and XbaI, and cloning it into cDNA cut with the same enzymes. The protein can be expressed using the methods that have been described herein for COMP/TSP-1 and COMP/TSP-2. Angiostatin, as it was isolated from mice bearing Lewis lung carcinoma, includes the first four kringle domains of plasminogen (amino acids 98-440) (O'Reilly, M.S., *et al., Cell 79*:315-328, 1994). It should be noted that smaller constructs that contain fewer kringle domains should also be active based on published data (Griscelli, F., *et al.*. *Proc. Natl. Acad. Sci. USA 95*:6367-6372, 1998). A 16,000 dalton fragment of prolactin and platelet factor 4 have also been reported to inhibit angiogenesis (Clapp, C. *et al., Endocrinology 133*:1292-1299, 1993; Gapta, S.K., *et al., Proc. Natl. Acad. Sci. USA 92*:7799-7803, 1995).

Also included in the inventions are compositions containing, as a biological ingredient, an anti-angiogenic chimeric protein, or a variant thereof to inhibit angiogenesis in mammalian tissues, and use of such compositions in the treatment of diseases and conditions characterized by, or associated with, angiogenic activity. Such methods can involve administration by oral, topical, injection, implantation, sustained release, or other delivery methods that bring one or more anti-angiogenic chimeric proteins in contact with cells whose growth is to be inhibited.

The present invention includes a method of treating an angiogenesis-mediated disease with a therapeutically effective amount of one or more anti-angiogenic chimeric proteins. Angiogenesis-mediated diseases can include, but are not limited to, cancers, solid tumors, tumor metastasis, benign tumors (*e.g.*, hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic

granulomas), rheumatoid arthritis, psoriasis, ocular angiogenic diseases (*e.g.*, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis), Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, and wound granulation.

"Cancer" means neoplastic growth, hyperplastic or proliferative growth or a pathological state of abnormal cellular development and includes solid tumors, non-solid tumors, and any abnormal cellular proliferation, such as that seen in leukemia. As used herein, "cancer" also means angiogenesis-dependent cancers and tumors, *i.e.*, tumors that require for their growth (expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying them with blood. "Regression" refers to the reduction of tumor mass and size. As used herein, the term "therapeutically effective amount" means the total amount of each active component of the composition or method that is sufficient to show a meaningful benefit to a treated human or other mammal, *i.e.*, treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. More specifically, for example, a therapeutically effective amount of an anti-angiogenic chimeric protein can cause a measurable reduction in the size or numbers of tumors, or in their rate of growth or multiplication, compared to untreated tumors. Other methods of assessing a "therapeutically effective amount," can include the result that blood vessel formation is measurably reduced in treated tissues compared to untreated tissues.

One or more anti-angiogenic chimeric proteins may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation, chemotherapy, or immunotherapy, combined with anti-angiogenic chimeric proteins, and then anti-angiogenic chimeric proteins may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

The compositions may further contain other agents which either enhance the activity of the protein or compliment its activity or use in treatment, such as

chemotherapeutic or radioactive agents. Such additional factors and/or agents may be included in the composition to produce a synergistic effect with protein of the invention, or to minimize side effects. Additionally, administration of the composition of the present invention may be administered concurrently with other therapies, *e.g.*, administered in conjunction with a chemotherapy, immunotherapy or radiation therapy regimen.

The angiogenesis-modulating composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage unit may be administered locally, for example at a tumor site, or may be implanted for systemic release of the angiogenesis-modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Examples of aerosol formulation include inhaler formulation for administration to the lungs.

The anti-angiogenic chimeric proteins can be provided as isolated and substantially purified proteins in pharmaceutically acceptable formulations (including aqueous or nonaqueous carriers or solvents) using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (*e.g.*, intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the anti-angiogenic chimeric proteins may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor, or implanted so that the anti-angiogenic chimeric proteins is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of anti-angiogenic chimeric proteins through cannulae to the site of interest, such as directly

into a growth or into the vascular supply to that growth. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.* (1991) (*J. Neurosurg.* 74:441-446), which is hereby incorporated by reference in its entirety.

As used herein, the terms "pharmaceutically acceptable," as it refers to compositions, carriers, diluents and reagents, represents that the materials are capable of administration to or upon a mammal with a minimum of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically, such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified, for example, in liposomes.

The dosage of the anti-angiogenic chimeric proteins of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The present invention also encompasses gene therapy whereby a polynucleotide encoding one or more anti-angiogenic chimeric proteins or one or more variants thereof, is introduced and regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in *Gene Transfer into Mammalian Somatic Cells in Vivo*, N. Yang (1992) *Crit. Rev. Biotechnol.* 12(4):335-356, which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line cells for use in either ex vivo or in vivo therapy. Gene therapy can function to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Strategies for treating these medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene for the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. For example, a gene encoding an anti-angiogenic chimeric protein may be inserted into tumor cells of a patient and thus inhibit angiogenesis.

Gene transfer methods for gene therapy fall into three broad categories: physical (*e.g.*, electroporation, direct gene transfer and particle bombardment), chemical (*e.g.*, lipid-based carriers, or other non-viral vectors) and biological (*e.g.*, virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using virally mediated gene transfer using a noninfectious virus to deliver the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun," may be used for in vitro insertion of anti-angiogenic chimeric proteins DNA or anti-angiogenic chimeric proteins regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to transfer the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of

the cell. Another chemical method uses receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and enveloping and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cells. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA.

Viral vectors have also been used to insert genes into cells using in vivo protocols. To direct the tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific can be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors have been widely utilized gene transfer vectors.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA complex can be conveniently introduced into body fluids or the bloodstream and then site-specifically directed to the target organ or

tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins, can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The gene therapy protocol for transfecting anti-angiogenic chimeric proteins into a patient may either be through integration of a gene encoding an anti-angiogenic chimeric protein into the genome of the cells, into minichromosomes or as a separate replicating or non-replicating DNA construct in the cytoplasm or nucleoplasm of the cell. Anti-angiogenic chimeric proteins expression may continue for a long-period of time or may be reinjected periodically to maintain a desired level of the anti-angiogenic chimeric proteins protein in the cell, the tissue or organ or a determined blood level.

EXAMPLES

Example 1: Construction of COMP/TSP-1 and COMP/TSP-2

The chimeric expression vectors have been produced from three distinct cDNAs. The first is a clone for human cartilage oligomeric matrix protein (COMP) and was isolated from a λgtll chondrocyte cDNA library (Doege, K.J, *et al., J. Biol. Chem. 266:*894-902 (1991)). This is an almost full-length clone for the COMP mRNA that only lacks a small region of the 5'-untranslated region. This clone (hCOMP-95) was used previously to determine the sequence of human COMP (GenBank Accession No. L32137; *Genomics, 24*:435-439 (1994)).

The second cDNA was produced using the polymerase chain reaction (PCR) with the human thrombospondin-1 (TSP-1) gene as the template. The TSP-1 clones were isolated from a human endothelial cell library (*J. Cell Biol. 103*:1635-1648

(1986)). The forward primer (GAT GAC GTC GAT GGT GGC TGG AGC CAC) (SEQ ID NO: 17) and the reverse primer (GAT CTA GAT TGG ACA GTC CTG CTT G) (SEQ ID NO: 18) produce a PCR product that is approximately 354 basepairs in size and has an Aat II restriction endonuclease site at the 5' end and an Xba I restriction endonuclease site at the 3' end. The PCR product encodes amino acids 417 to 530 and includes the second and third type 1 repeats of TSP-1 (see Figure 1 for the numbering of amino acids in TSP-1). The coding sequence for the first type 1 repeat was not included in the PCR product, by design, because it contains an RFK sequence that has been shown to activate TGF-β. This activity is not required to inhibit angiogenesis and it may produce unwanted secondary effects on numerous cell types. Vectors that include the first type 1 repeat can be constructed, using the same approach, if this region is found to enhance the antiangiogenic activity or other activities.

The third cDNA was produced by PCR with a human heart cDNA library (catalog no. 936208 from Stratagene, LaJolla, CA) as the template. The forward primer (GAT GAC GTC GAG GAG GGC TGG TCT CCG) (SEQ ID NO: 19) and the reverse primer (GAT CTA GAC ACG GGG CAG CTC CTC TTG) (SEQ ID NO: 20) produced a PCR product that is approximately 520 base pairs in size and has an Aat II restriction endonuclease site at the 5' end and an Xba I restriction endonuclease site at the 3' end. The PCR product codes for amino acids 381 to 550 of TSP-2 and, includes all three type 1 repeats of TSP-2 (see Figure 2 for numbering of amino acids in TSP-2). The sequence of the PCR primers was based on the human TSP-2 sequence in the GenBank database (Accession No. L12350). The sequences of the PCR products were determined to establish that mutations that affect the amino acid sequence had not been introduced during the PCR.

The COMP/TSP-1 and COMP-TSP-2 expression vectors were constructed by cutting the PCR products with Aat II and Xba I and subcloning them into the COMP cDNA vector [derived from Bluescript (Stratagene, La Jolla, CA)] cut with the same enzymes. The portion of COMP that was retained includes the signal sequence, the regions required for pentamerization and the first type 2 repeat (amino acids 1 to 128 on the enclosed sequence; Figure 3). Since there was an internal Aat

II site in the TSP-2 PCR product, it had to be cloned into the vector in two steps. A 430 basepair Aat II/Xba I fragment of the TSP-2 PCR product was subcloned into the vector containing the portion of COMP as a first step. The resulting subclone was cut with Aat II, and a 90 base pair Aat II fragment of the PCR product was ligated into the expression vector. The final forms of the cDNAs were confirmed to have the predicted structure by nucleotide sequencing. They were then cut with Eco R1 and Xba I and ligated into the pcDNA 3.1 (Invitrogen; Carlsbad, CA) vector cut with the same enzymes. The DNA sequences of COMP/TSP-1 and COMP/TSP-2 are shown in Figures 4A and 4B and Figures 5A and 5B, respectively. The predicted molecular weights of the subunits of COMP/TSP-1 and COMP/TSP-2 should be approximately 24,200 and 30,000, respectively. The fully assembled COMP/TSP-1 and COMP/TSP-2 proteins should be 121,000 Da and 150,000 Da, respectively. The amino acid sequences of these proteins are shown in Figures 4A and 4B and Figures 5A and 5B, respectively.

Example 2: Production of Isolated COMP/TSP-1 and COMP/TSP-2

To express these chimeric proteins, the expression vectors can be transfected into human kidney 293 cells using the Lipofectin protocol (Gibco Laboratories). The cells can be selected with Zeocin and individual clones can be grown. The secretion of COMP/TSP-1 and COMP/TSP-2 can be monitored with western blotting using polyclonal antibodies to the region of COMP that is present in both expressed proteins. These antibodies have been produced by immunizing rabbits with a synthetically produced peptide, having an amino acid sequence derived from the N-terminal end of COMP, linked to a carrier protein. The amino acid sequence of the peptide is: SDLGPQMLRELQETN (SEQ ID NO: 21). A clone that expresses high levels of the protein can be grown in large volume flasks and in serum free media.

Example 3: Inhibition of Tumor Growth by COMP/TSP-1

A cDNA of thrombospondin-1 (TSP-1) containing the second and third type-1 repeats and the COMP assembly sequence (COMP/TSP-1) was produced by PCR

using constructs derived as above as template, and was cloned into the expression vector pNeo (Invitrogen, Carlsbad, CA). Both the resulting COMP/TSP-1 construct and the unaltered vector alone were transfected into the human squamous carcinoma cell line A431 (Streit, M., *et al., American Journal of Pathology 155*:441-452, 1999), and positive clones were selected using Geneticin at a concentration of 800 μg/ml. The growth curves of positive clones were determined over an 8 day period. Clones of pNeo- and COMP/TSP-1 construct-transfected cells that had similar growth curves were selected to test the effect of the chimeric protein on tumor growth in nude mice. A total of five mice pre group were injected intradermally at the shoulders with 5×10^6 cells per site, two sites per mouse. Every week the tumors were measured with calipers. At three weeks, the mice were sacrificed and the tumors were removed for further studies. As can be seen from Figure 7, expression of COMP/TSP-1 caused inhibition of the growth of the tumors in this model.

All references (e.g., journal articles, books, published patent applications and patents, etc.) cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A nucleic acid molecule encoding a chimeric protein comprising the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

2. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

3. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

4. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1.

5. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

6. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human endostatin, wherein the chimeric protein has anti-angiogenic activity.

7. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human angiostatin, wherein the chimeric protein has anti-angiogenic activity.

8. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human prolactin, wherein the chimeric protein has anti-angiogenic activity.

9. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human platelet factor 4, wherein the chimeric protein has anti-angiogenic activity.

10. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region, and the first, second, and third type 1 repeats of human TSP-1.

11. A nucleic acid molecule encoding a protein having the amino acid sequence SEQ ID NO: 5.

12. A vector comprising nucleic acid encoding a chimeric protein comprising the second and third type 1 repeats of human TSP-1 but not the TGF-β activation region of human TSP-1.

13. A host cell comprising the vector of Claim 12.

14. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

15. A host cell comprising the vector of Claim 14.

16. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, said method comprising maintaining the host cell of Claim 15 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

17. The method of Claim 16 further comprising isolating the chimeric protein.

18. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

19. A host cell comprising the vector of Claim 18.

20. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1, said method comprising maintaining the host cell of Claim 19 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

21. The method of Claim 20 further comprising isolating the chimeric protein.

22. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region, and the first, second, and third type 1 repeats of human TSP-1.

23. A vector comprising nucleic acid encoding a protein having the amino acid sequence SEQ ID NO: 5.

24. A host cell comprising the vector of Claim 23.

25. A chimeric protein comprising the second and third type 1 repeat of human TSP-1, but not the TGF-β activation region of human TSP-1.

26. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

27. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

28. A chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1.

29. A chimeric protein comprising the multimerization domain of human COMP and a portion of human endostatin, wherein the chimeric protein has anti-angiogenic activity.

30. A chimeric protein comprising the multimerization domain of human COMP and a portion of human angiostatin, wherein the chimeric protein has anti-angiogenic activity.

31. A chimeric protein comprising the multimerization domain of human COMP and a portion of human prolactin, wherein the chimeric protein has anti-angiogenic activity.

32. A chimeric protein comprising the multimerization domain of human COMP and a portion of human platelet factor 4, wherein the chimeric protein has anti-angiogenic activity.

33. A protein having the amino acid sequence SEQ ID NO: 5.

34. An isolated nucleic acid molecule encoding a chimeric protein comprising the three type 1 repeats of human TSP-2.

35. A vector comprising nucleic acid encoding a chimeric protein comprising the three type 1 repeats of human TSP-2.

36. A host cell comprising the vector of Claim 35.

37. A method for producing a chimeric protein which comprises the three type 1 repeats of human TSP-2, said method comprising maintaining the host cell of Claim 36 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

38. The method of Claim 37 further comprising isolating the chimeric protein.

39. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

40. A vector comprising isolated nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

41. A host cell comprising the vector of Claim 40.

42. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2, said method comprising maintaining the host cell of Claim 41 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

43. The method of Claim 42 further comprising isolating the chimeric protein.

44. A nucleic acid molecule encoding a protein having the amino acid sequence SEQ ID NO: 7.

45. A vector comprising nucleic acid encoding a protein having the amino acid sequence SEQ ID NO: 7.

46. A host cell comprising the vector of Claim 45.

47. A chimeric protein comprising the three type 1 repeats of human TSP-2.

48. A chimeric protein comprising the procollagen homology region of TSP-2 and the three type 1 repeats of human TSP-2.

49. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

50. A protein having the amino acid sequence SEQ ID NO: 7.

51. A method for inhibiting angiogenesis in a human or other mammal, the method comprising administering to the human or other mammal a therapeutically effective amount of an anti-angiogenic chimeric protein.

52. The method of Claim 51 wherein the anti-angiogenic chimeric protein is selected from the group consisting of:

a) a chimeric protein comprising the second and third type 1 repeats of human TSP-1;

b) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1;

c) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1;

d) a chimeric protein comprising the multimerization domain of human COMP, the procollagen region, and the first, second, and third type 1 repeats of human TSP-1; and

e) a chimeric protein comprising the three type 1 repeats of human TSP-2; and (6) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

53. The method of Claim 51 wherein the anti-angiogenic protein is administered locally at the site of one or more growths.

human thrombospondin-1

NH2
1 NRIPESGGDNSVFDIFELTGAARKGSGRRLVKGPDPSSPAFRIEDANLIPPVPDDKFQDL
61 VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHSGQVFSVVSNGKAGTLDLSLTVQGKQ
121 HVVSVEEALLATGQWKSITLFVQEDRAQLYIDCEKMENAELDVPIQSVFTRDLASIARLR
181 IAKGGVNDNFQGVLQNVRFVFGTTPEDILRNKGCSSSTSVLLTLDNNVVNGSSPAIRTNY

241 IGHKTKDLQAICGISCDELSSM

P
263 VLELRGLRTIVTTLQDSIRKVTEENKELANELRRPPLCYHNGVQYRNNE
312 EWTVDSCTECHCQNSVTICKKVSCPIMPCSNATVPDGECCPRCWPSDSA

type 1
361 DDGWSPWSEWTSCSTSCGNGIQQRGRSCDSLNNR-----CEGSSVQTRTCHIQECDKRFKQ
417 DGGWSHWSPWSSCSVTCGDGVITRIRLCNSPSPQMNGKPCEGEARETKACKKDACPI
474 NGGWGPWSPWDICSVTCGGGVQKRSRLCNNPTPQFGGKDCVGDVTENQICNKQDCPI

type 2
531 DGCLSNP--CFAGV---KCT---SYPDGSWKCGACPPGYSG-------------NGIQCTDV
572 DECKEVPDACFNHNGEHRCEN----TDPGYNCLPCPPRFTGSQPFGQGVEHATANKQVCKPR
630 NPCTDGTHDCNKNA---KCNYLGHYSDPMYRC-ECKPGYAG-------------NGIICGE

674 DTDLDGWPNENLVCVANATYHCKK

type 3
698 DNCPNLPNSGQEDYDKDGIGDACDD--DDDNDKIPDDR
734 DNCPFHYNPAQYDYDRDDVGDRC
757 DNCPYNHNPDQADTDNNGEGDACAA--DIDGDGILNER
793 DNCQYVYNVDQRDTDMDGVGDQC
816 DNCPLEHNPDQLDSDSDRIGDTCDNNQDIDEDGHQNNL
854 DNCPYVPNANQADHDKDGKGDACDH--DDDNDGIPDDK
890 DNCRLVPNPDQKDSDGDGRGDACKD--DFDHDSVPDID

COOH
926 DICPENVDISETDFRRFQMIPLDPKGTSQNDPNWVVRHQGKELVQTVNCDPGLAVGYDEF
986 NAVDFSGTFFINTERDDDYAGFVFGYQSSSRFYVVMWKQVTQSYWDTNPTRAQGYSGLSV
1046 KVVNSTTGPGEHLRNALWHTGNTPGQVRTLWHDPRHIGWKDFTAYRWRLSHRPKTGFIRV
1106 VHYEGKKIMADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYECRDP

FIG. 1

human thrombospondin-2

NH_2	1	MVWRLVLLALWVWPSTQAGHQDKDTTFDLFSISNINRKTIGAKQFRGPDPGVPAYRFVRF
	61	DYIPPVNADDLSKITKIMRQKEGFFLTAQLKQDGKSRGTLLALEGPGLSQRQFEIVSNGP
	121	ADTLDLTYWIDGTRHVVSLEDVGLADSQWKNVTVQVAGETYSLHVGCDLIGPVALDEPFY
	181	EHLQAEKSRMYVAKGSARESHFRGLLQNVHLVFENSVEDILSKKGCQQGQGAEINAISEN
	241	TETLRLGPHVTTEYVGPSSERRPEVCERSCEELGNM
P	277	VQELSGLHVLVNQLSENLKRVSNDNQFLWELIGGPPKTRNMSACWQDGRFFAEHE
	332	TWVVDSCTTCTCKKFKTICHQITCPPATCASPSFVEGECCPSCLHSVDG
type 1	381	EEGWSPWAEWTQCSVTCGSGTQQRGRSCDVTSNT-----CLGPSIQTRACSLSKCDTRIRQ
	437	DGGWSHWSPWSSCSVTCGVGNITRIRLCNSPVPQMGGKNCKGSGRETKACQGAPCPI
	494	DGRWSPWSPWSACTVTCAGGIRERTRVCNSPEPQYGGKACVGDVQERQMCNKRSCPV
type 2	551	DGCLSNP--CFPGA---QCS---SFPDGSWSCGFCPVGFLG-------------NGTHCEDL
	592	DECALVPDICFSTSKVPRCVN----TQPGFHCLPCPPRYRGNQPVGVGLEAAKTEKQVCEPE
	650	NPCKDKTHNCHKHA---ECIYLGHFSDPMYKCE-CQTGYAG-------------DGLICGE
	694	DSDLDGWPNLNLVCATNATYHCIK
	718	DNCPHLPNSGQEDFDKDGIGDACDD--DDDNDGVTDEK
	748	DNCQLLFNPRQADYDKDEVGDRC
type 3	777	DNCPYVHNPAQIDTDNNGEGDACSV--DIDGDDVFNER
	813	DNCPYVYNTDQRDTDGDGVGDHC
	836	DNCPLVHNPDQTDVDNDLVGDQCDNNEDIDDDGHQNNQ
	874	DNCPYISNANQADHDRDGQGDACDP--DDDNDGVPDDR
	910	DNCRLVFNPDQEDLDGDGRGDICKD--DFDNDNIPDID
COOH	946	DVCPENNAISETDFRNFQMVPLDPKGTTQIDPNWVIRHQGKELVQTANSDPGIAVGFDEF
	1006	GSVDFSGTFYVNTDRDDDYAGFVFGYQSSSRFYVVMWKQVTQTYWEDQPTRAYGYSGVSL
	1066	KVVNSTTGTGEHLRNALWHTGNTPGQVRTLWHDPRNIGWKDYTAYRWHLTHRPKTGYIRV
	1126	LVHEGKQVMADSGPIYDQTYAGGRLGLFVFSQEMVYFSDLKYECRDI

FIG. 2

```
human COMP

          1  MVPDTACVLLLTLAALGASGQGQSPLGSDLGPQMLRELQETNAALQDVRDWLRQQVREIT

         61  FLKNTVMECDACGMQQSVRTGLPSVRPL

         89  LHCAPGF---CFPGVACIQTESGGR-CGPCPAGPTG-------------NGSHCTDV
        129  NECNAHP---CFPRVRCINTSPGFR-CEACPPGYSGPTHQGVGLAFAKANKQVCTDI
type 2  182  NECETGQHN-CVPNSVCINTRGSFQ-CGPCQPGFVG-------------DQASGCQRGAQ
        227  RFCPDGSPSECHEHADCVLERDGSRSCV-CRVGWAG-------------NGILCGR

        269  DTDLDGFPDEKLRCPEPQCRK

        290  DNCVTVPNSGQEDVDRDGIGDACD--PDADGDGVPNEK
        326  DNCPLVRNPDQRNTDEDKWGDAC
        349  DNCRSQKNDDQKDTDQDGRGDACD--DDIDGDRIRNQA
type 3  385  DNCPRVPNSDQKDSDGDGIGDAC
        408  DNCPQKSNPDQADVDHDFVGDACDSDQDQDGDGHQDSR
        446  DNCPTVPNSAQEDSDHDGQGDACD--DDDDNDGVPDSR
        482  DNCRLVPNPGQEDADRDGVGDVCQ--DDFDADKVVDKI

        518  DVCPENAEVTLTDFRAFQTVVLDPEGDAQIDPNWVVLNQGREIVQTMNSDPGLAVGYTAF
COOH    578  NGVDFEGTFHVNTVTDDDYAGFIFGYQDSSSFYVVMWKQMEQTYWQANPFRAVAEPGIQL
        638  KAVKSSTGPGEQLRNALWHTGDTESQVRLLWKDPRNVGWKDKKSYRWFLQHRPQVGYIRV
        698  RFYEGPELVADSNVVLDTTMRGGRLGVFCFSQENIIWANLRYRCNDTIPEDYETHQLRQA
```

FIG. 3

```
CAGCACCCAG CTCCCCGCCA CCGCC ATG GTC CCC GAC ACC GCC TGC GTT CTT      52
                            Met Val Pro Asp Thr Ala Cys Val Leu
                             1               5

CTG CTC ACC CTG GCT GCC CTC GGC GCG TCC GGA CAG GGC CAG AGC CCG     100
Leu Leu Thr Leu Ala Ala Leu Gly Ala Ser Gly Gln Gly Gln Ser Pro
 10                  15                  20                  25

TTG GGC TCA GAC CTG GGC CCG CAG ATG CTT CGG GAA CTG CAG GAA ACC     148
Leu Gly Ser Asp Leu Gly Pro Gln Met Leu Arg Glu Leu Gln Glu Thr
                 30                  35                  40

AAC GCG GCG CTG CAG GAC GTG CGG GAC TGG CTG CGG CAG CAG GTC AGG     196
Asn Ala Ala Leu Gln Asp Val Arg Asp Trp Leu Arg Gln Gln Val Arg
             45                  50                  55

GAG ATC ACG TTC CTG AAA AAC ACG GTG ATG GAG TGT GAC GCG TGC GGG     244
Glu Ile Thr Phe Leu Lys Asn Thr Val Met Glu Cys Asp Ala Cys Gly
         60                  65                  70

ATG CAG CAG TCA GTA CGC ACC GGC CTA CCC AGC GTG CGG CCC CTG CTC     292
Met Gln Gln Ser Val Arg Thr Gly Leu Pro Ser Val Arg Pro Leu Leu
     75                  80                  85

CAC TGC GCG CCC GGC TTC TGC TTC CCC GGC GTG GCC TGC ATC CAG ACG     340
His Cys Ala Pro Gly Phe Cys Phe Pro Gly Val Ala Cys Ile Gln Thr
 90                  95                 100                 105

GAG AGC GGC GGC CGC TGC GGC CCC TGC CCC GCG GGC TTC ACG GGC AAC     388
Glu Ser Gly Gly Arg Cys Gly Pro Cys Pro Ala Gly Phe Thr Gly Asn
                110                 115                 120
```

FIG 4A

```
GGC TCG CAC TGC ACC GAC GTC GAT GGT GGC TGG AGC CAC TGG TCC CCG     436
Gly Ser His Cys Thr Asp Val Asp Gly Gly Trp Ser His Trp Ser Pro
            125                 130                 135

TGG TCA TCT TGT TCT GTG ACA TGT GGT GAT GGT GTG ATC ACA AGG ATC     484
Trp Ser Ser Cys Ser Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile
        140                 145                 150

CGG CTC TGC AAC TCT CCC AGC CCC CAG ATG AAC GGG AAA CCC TGT GAA     532
Arg Leu Cys Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu
    155                 160                 165

GGC GAA GCG CGG GAG ACC AAA GCC TGC AAG AAA GAC GCC TGC CCC ATC     580
Gly Glu Ala Arg Glu Thr Lys Ala Cys Lys Lys Asp Ala Cys Pro Ile
170                 175                 180                 185

AAT GGA GGC TGG GGT CCT TGG TCA CCA TGG GAC ATC TGT TCT GTC ACC     628
Asn Gly Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr
                190                 195                 200

TGT GGA GGA GGG GTA CAG AAA CGT AGT CGT CTC TGC AAC AAC CCC ACA     676
Cys Gly Gly Gly Val Gln Lys Arg Ser Arg Leu Cys Asn Asn Pro Thr
            205                 210                 215

CCC CAG TTT GGA GGC AAG GAC TGC GTT GGT GAT GTA ACA GAA AAC CAG     724
Pro Gln Phe Gly Gly Lys Asp Cys Val Gly Asp Val Thr Glu Asn Gln
        220                 225                 230

ATC TGC AAC AAG CAG GAC TGT CCA ATC TAG  A                          755
Ile Cys Asn Lys Gln Asp Cys Pro Ile  *
    235                 240
```

FIG. 4B

```
CAGCACCCAG CTCCCCGCCA CCGCC ATG GTC CCC GAC ACC GCC TGC GTT CTT      52
                            Met Val Pro Asp Thr Ala Cys Val Leu
                             1               5

CTG CTC ACC CTG GCT GCC CTC GGC GCG TCC GGA CAG GGC CAG AGC CCG     100
Leu Leu Thr Leu Ala Ala Leu Gly Ala Ser Gly Gln Gly Gln Ser Pro
 10                  15                  20                  25

TTG GGC TCA GAC CTG GGC CCG CAG ATG CTT CGG GAA CTG CAG GAA ACC     148
Leu Gly Ser Asp Leu Gly Pro Gln Met Leu Arg Glu Leu Gln Glu Thr
                 30                  35                  40

AAC GCG GCG CTG CAG GAC GTG CGG GAC TGG CTG CGG CAG CAG GTC AGG     196
Asn Ala Ala Leu Gln Asp Val Arg Asp Trp Leu Arg Gln Gln Val Arg
             45                  50                  55

GAG ATC ACG TTC CTG AAA AAC ACG GTG ATG GAG TGT GAC GCG TGC GGG     244
Glu Ile Thr Phe Leu Lys Asn Thr Val Met Glu Cys Asp Ala Cys Gly
         60                  65                  70

ATG CAG CAG TCA GTA CGC ACC GGC CTA CCC AGC GTG CGG CCC CTG CTC     292
Met Gln Gln Ser Val Arg Thr Gly Leu Pro Ser Val Arg Pro Leu Leu
     75                  80                  85

CAC TGC GCG CCC GGC TTC TGC TTC CCC GGC GTG GCC TGC ATC CAG ACG     340
His Cys Ala Pro Gly Phe Cys Phe Pro Gly Val Ala Cys Ile Gln Thr
 90                  95                 100                 105

GAG AGC GGC GGC CGC TGC GGC CCC TGC CCC GCG GGC TTC ACG GGC AAC     388
Glu Ser Gly Gly Arg Cys Gly Pro Cys Pro Ala Gly Phe Thr Gly Asn
                110                 115                 120

GGC TCG CAC TGC ACC GAC GTC GAG GAG GGC TGG TCT CCG TGG GCA GAG     436
Gly Ser His Cys Thr Asp Val Glu Glu Gly Trp Ser Pro Trp Ala Glu
            125                 130                 135

TGG ACC CAG TGC TCC GTG ACG TGT GGC TCT GGG ACC CAG CAG AGA GGC     484
Trp Thr Gln Cys Ser Val Thr Cys Gly Ser Gly Thr Gln Gln Arg Gly
        140                 145                 150
```

FIG. 5A

```
CGG TCC TGT GAC GTC ACC AGC AAC ACC TGC TTG GGG CCC TCG ATC CAG    532
Arg Ser Cys Asp Val Thr Ser Asn Thr Cys Leu Gly Pro Ser Ile Gln
    155                 160                 165

ACA CGG GCT TGC AGT CTG AGC AAG TGT GAC ACC CGC ATC CGG CAG GAC    580
Thr Arg Ala Cys Ser Leu Ser Lys Cys Asp Thr Arg Ile Arg Gln Asp
170                 175                 180                 185

GGC GGC TGG AGC CAC TGG TCA CCT TGG TCT TCA TGC TCT GTG ACC TGT    628
Gly Gly Trp Ser His Trp Ser Pro Trp Ser Ser Cys Ser Val Thr Cys
                190                 195                 200

GGA GTT GGC AAT ATC ACA CGC ATC CGT CTC TGC AAC TCC CCA GTG CCC    676
Gly Val Gly Asn Ile Thr Arg Ile Arg Leu Cys Asn Ser Pro Val Pro
            205                 210                 215

CAG ATG GGG GGC AAG AAT TGC AAA GGG AGT GGC CGG GAG ACC AAA GCC    724
Gln Met Gly Gly Lys Asn Cys Lys Gly Ser Gly Arg Glu Thr Lys Ala
        220                 225                 230

TGC CAG GGC GCC CCA TGC CCA ATC GAT GGC CGC TGG AGC CCC TGG TCC    772
Cys Gln Gly Ala Pro Cys Pro Ile Asp Gly Arg Trp Ser Pro Trp Ser
    235                 240                 245

CCG TGG TCG GCC TGC ACT GTC ACC TGT GCC GGT GGG ATC CGG GAG CGC    820
Pro Trp Ser Ala Cys Thr Val Thr Cys Ala Gly Gly Ile Arg Glu Arg
250                 255                 260                 265

ACC CGG GTC TGC AAC AGC CCT GAG CCT CAG TAC GGA GGG AAG GCC TGC    868
Thr Arg Val Cys Asn Ser Pro Glu Pro Gln Tyr Gly Gly Lys Ala Cys
            270                 275                 280

GTG GGG GAT GTG CAG GAG CGT CAG ATG TGC AAC AAG AGG AGC TGC CCC    916
Val Gly Asp Val Gln Glu Arg Gln Met Cys Asn Lys Arg Ser Cys Pro
            285                 290                 295

GTG TCT AGA                                                        925
Val Ser Arg
```

FIG. 5B

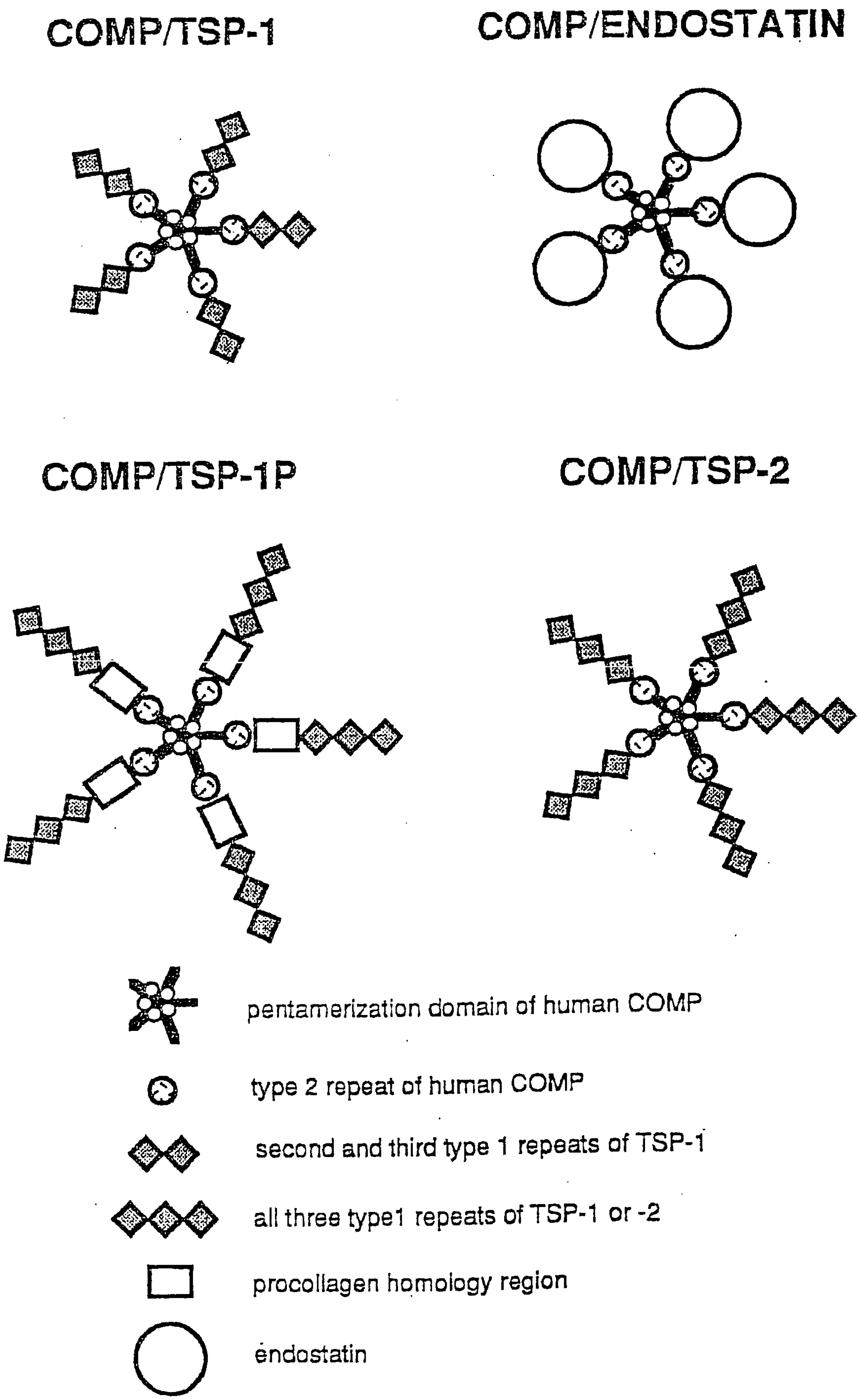
COMP/TSP-1
COMP/ENDOSTATIN
COMP/TSP-1P
COMP/TSP-2
pentamerization domain of human COMP
type 2 repeat of human COMP
second and third type 1 repeats of TSP-1
all three type1 repeats of TSP-1 or -2
procollagen homology region
endostatin

FIG. 6

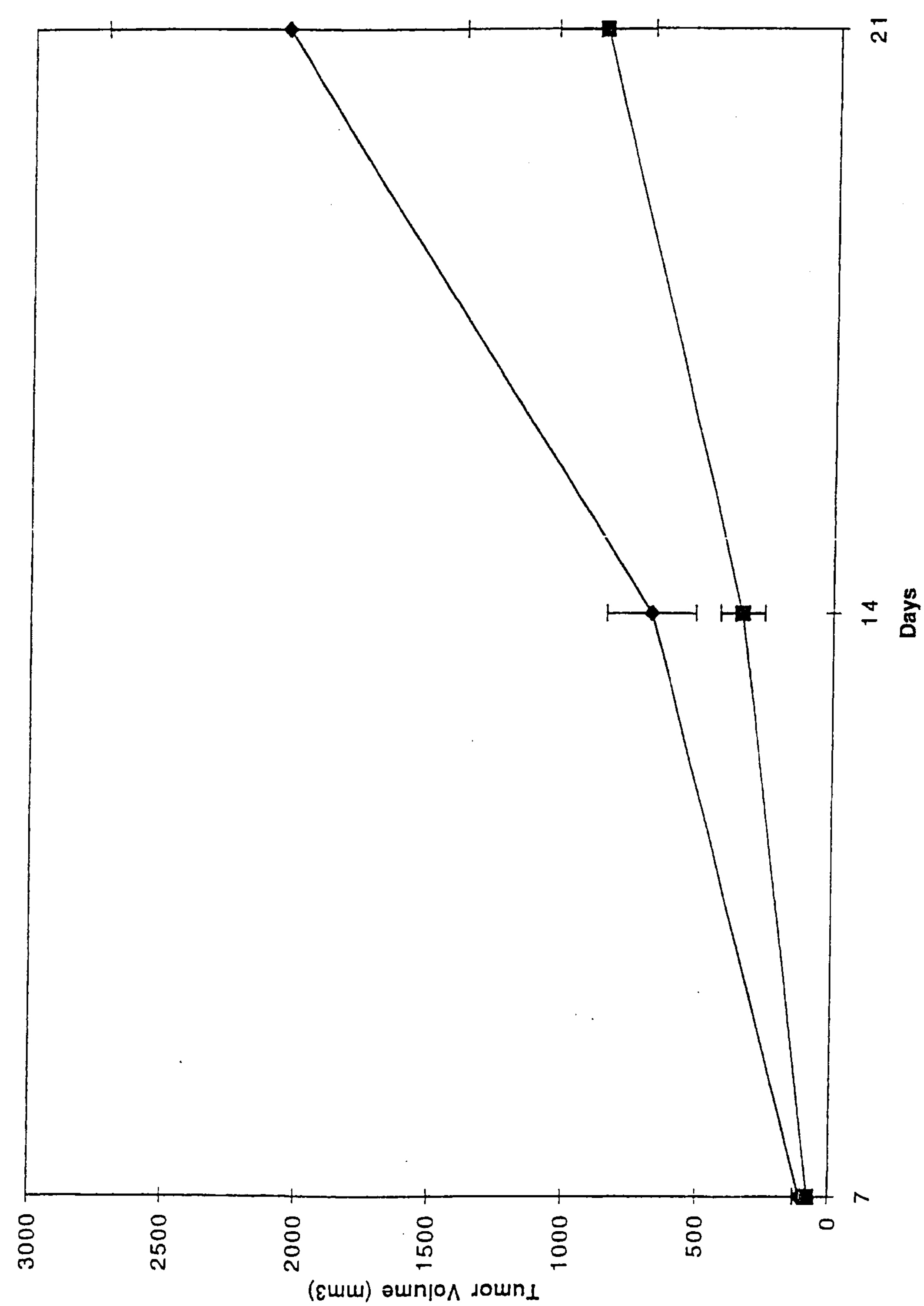

FIG. 7

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60/118,053 1 February 1999 (01.02.1999) US

(71) Applicant (*for all designated States except US*): **BETH ISRAEL DEACONESS MEDICAL CENTER** [US/US]; 330 Brookline Avenue, Boston, MA 02215 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): **LAWLER, John, W.** [US/US]; 6 Gale Road, Swampscott, MA 01907 (US).

(74) Agents: **HOGLE, Doreen, M.** et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

(81) Designated States (*national*): AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published:
— *With international search report.*

(88) Date of publication of the international search report: 15 February 2001

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

WO 00/44908 A3

(54) Title: COMP/TSP-1, COMP/TSP-2 AND OTHER TSP CHIMERIC PROTEINS

(57) Abstract: Tumors attract blood vessels in order to grow by a process called angiogenesis. The relative quantity of stimulators and inhibitors is an important determining factor for the initiation of angiogenesis. Thrombospondins-1 and -2 are adhesive glycoproteine that have the ability to inhibit aniogenesis. This inhibiting activity has been mapped to the type 1 repeats of TSP-1 and TSP-2. The invention includes chimeric proteins that contain anti-angiogenic portions of TSP-1, TSP-2, endostatin, angiostatin, platelet factor 4, or prolactin, linked to a portion of the N-terminal region of human cartilage oligomeric matrix protein (COMP) that allows formation of pentamers. Also described herein are the nucleic acid molecules, vectors, and host cells for expressing and producing these chimeric proteins. Further embodiments of the invention include methods to treat humans or other mammals with anti-angiogenic proteins to reduce tumor size or rate of growth. Since the type 1 repeat region of TSP-1 and TSP-2 reportedly inhibits HIV infection, chimeric proteins comprising these repeats may also be used for this purpose, as well as to inhibit angiogenesis.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02482

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C07K14/78 C07K19/00 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, MEDLINE, SCISEARCH, BIOTECHNOLOGY ABS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PANETTI TS ET AL: "Interaction of recombinant procollagen and properdin modules of thrombospondin-1 with heparin and fibrinogen/fibrin." JOURNAL OF BIOLOGICAL CHEMISTRY, JAN 1 1999, 274 (1) P430-7, XP002140107 UNITED STATES	1,12,13, 25
A	abstract; figure 2B	2-5,10, 11, 14-24, 26-28

	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	18. 10. 00

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Gurdjian, D

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INTERNATIONAL SEARCH REPORT

Intern nal Application No
PCT/US 00/02482

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	QABAR AZIZ N ET AL: "A chimeric murine TSP3/human TSP1 is a pentamer with abolished antiangiogenic activity." ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS ON EXPERIMENTAL BIOLOGY 97;NEW ORLEANS, LOUISIANA, USA; APRIL 6-9, 1997, vol. 11, no. 3, 1997, page A63 XP000914726 FASEB Journal 1997 ISSN: 0892-6638 the whole document ---	1,12,13, 25
A	WO 98 18943 A (CIBA GEIGY AG ;KAJAVA ANDREY (CH); UNIV LAUSANNE (CH); CRAMERI RET) 7 May 1998 (1998-05-07) abstract; claims 1-32 ---	1-5, 10-28,33
A	WO 98 39418 A (ARIAD GENE THERAPEUTICS INC ;GILMAN MICHAEL Z (US)) 11 September 1998 (1998-09-11) claims 1-5 ---	1-5, 10-28,33
A	WO 93 16716 A (UNIV NORTHWESTERN) 2 September 1993 (1993-09-02) claims 1-11; figures SEQ.ID.1,2 ---	1-5, 10-28,33
A	KUNO ET AL: "Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 1, 3 January 1997 (1997-01-03), pages 556-562, XP002076038 ISSN: 0021-9258 abstract; figures 5,6 ---	1-5, 10-28,33
Y	WO 96 37621 A (HOESS ADOLF ;PACK PETER (DE); MORPHOSYS PROTEINOPTIMIERUNG (DE)) 28 November 1996 (1996-11-28) claims 1,4-7 ---	6-9, 29-32
Y	EP 0 407 122 A (REPLIGEN CORP) 9 January 1991 (1991-01-09) claims 1-23 ---	9,32
Y	WO 98 54217 A (CHILDRENS MEDICAL CENTER) 3 December 1998 (1998-12-03) abstract; claims 1-15 ---	7,30
	-/--	

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INTERNATIONAL SEARCH REPORT

Interr. ıal Application No
PCT/US 00/02482

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 51323 A (STRUMAN INGRID ;MARTIAL JOSEPH A (BE); TAYLOR ROBERT (US); UNIV CA) 19 November 1998 (1998-11-19) abstract; claims 1-26 ---	8,31
Y	NGUYEN JOSEPHINE T ET AL: "Adeno-associated virus-mediated delivery of antiangiogenic factors as an antitumor strategy." CANCER RESEARCH, vol. 58, no. 24, 15 December 1998 (1998-12-15), pages 5673-5677, XP000857408 ISSN: 0008-5472 the whole document ---	6,29
A	TOMSCHY ANDREA ET AL: "Homophilic adhesion of E-cadherin occurs by a co-operative two-step interaction of N-terminal domains." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 15, no. 14, 1996, pages 3507-3514, XP002147181 ISSN: 0261-4189 the whole document ---	6-9, 29-32
A	TERSKIKH ALEXEY V ET AL: ""Peptabody": A new type of high avidity binding protein." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 5, 1997, pages 1663-1668, XP002147182 1997 ISSN: 0027-8424 the whole document ---	6-9, 29-32
A	NEWTON GAIL ET AL: "Characterization of human and mouse cartilage oligomeric matrix protein." GENOMICS, vol. 24, no. 3, 1994, pages 435-439, XP002147953 ISSN: 0888-7543 the whole document ---	34-50
A	O'ROURKE KM ET AL: "Thrombospondin 1 and thrombospondin 2 are expressed as both homo- and heterotrimers." JOURNAL OF BIOLOGICAL CHEMISTRY, DEC 15 1992, 267 (35) P24921-4, XP002147183 UNITED STATES the whole document -----	34-50

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INTERNATIONAL SEARCH REPORT

International application No
PCT/US 00/02482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely.

Although claims 51-53 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 51-53 partially and 1-5,10-28 ,33

   TSP-1 chimeric protein constructs, corresponding nucleic acids ,vectors ,host cells , method of producing chimeric proteins

2. Claims: 51-53 partially and 34-50

   TSP-2 chimeric protein constructs, corresponding nucleic acids ,vectors ,host cells ,method of producing chimeric proteins

3. Claims: 51-53 partially and 6-9, 29-32

   COMP chimeric protein constructs, not provided in the first and second invention of present application , corresponding nucleic acids ,vectors ,host cells , method of producing chimeric proteins

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern al Application No
PCT/US 00/02482

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9818943	A	07-05-1998	AU	6909298	A	22-05-1998
			EP	0938571	A	01-09-1999
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			CA	2019086	A	06-01-1991
			DE	69028739	D	07-11-1996
			DE	69028739	T	13-02-1997
			DK	407122	T	17-02-1997
			EP	0723015	A	24-07-1996
			ES	2092493	T	01-12-1996
			GR	3021658	T	28-02-1997
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			AU	7704998	A	30-12-1998
			EP	0996632	A	03-05-2000
WO 9851323	A	19-11-1998	AU	7383298	A	08-12-1998

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(22) International Filing Date: 1 February 2000 (01.02.2000)

(25) Filing Language: English

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(30) Priority Data:
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(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **LAWLER, John, W.** [US/US]; 6 Gale Road, Swampscott, MA 01907 (US).

(74) Agents: **HOGLE, Doreen, M.** et al.; Hamilton, Brook, Smith & Reynolds, P.C., Two Militia Drive, Lexington, MA 02421 (US).

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(54) Title: COMP/TSP-1, COMP/TSP-2 AND OTHER TSP CHIMERIC PROTEINS

(57) Abstract: Tumors attract blood vessels in order to grow by a process called angiogenesis. The relative quantity of stimulators and inhibitors is an important determining factor for the initiation of angiogenesis. Thrombospondins-1 and -2 are adhesive glycoproteine that have the ability to inhibit aniogenesis. This inhibiting activity has been mapped to the type 1 repeats of TSP-1 and TSP-2. The invention includes chimeric proteins that contain anti-angiogenic portions of TSP-1, TSP-2, endostatin, angiostatin, platelet factor 4, or prolactin, linked to a portion of the N-terminal region of human cartilage oligomeric matrix protein (COMP) that allows formation of pentamers. Also described herein are the nucleic acid molecules, vectors, and host cells for expressing and producing these chimeric proteins. Further embodiments of the invention include methods to treat humans or other mammals with anti-angiogenic proteins to reduce tumor size or rate of growth. Since the type 1 repeat region of TSP-1 and TSP-2 reportedly inhibits HIV infection, chimeric proteins comprising these repeats may also be used for this purpose, as well as to inhibit angiogenesis.

COMP/TSP-1, COMP/TSP-2 AND OTHER CHIMERIC PROTEINS

RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/118,053 filed February 1, 1999, the entire teachings of which are incorporated herein by reference.

BACKGROUND OF THE INVENTION

Thrombospondins are a family of calcium-binding multifunctional glycoproteins that are secreted by various cell types and are developmentally regulated components of the extracellular matrix (Bornstein, P., *FASEB J., 6:*3290-3299, 1992; Bornstein, P., *J. Cell Biol., 130:*503-506, 1995). Among their functions are modulating cell attachment, migration and proliferation.

One member of this family, cartilage oligomeric matrix protein (COMP) is a pentamer in which multimerization appears to be directed by α-helical segments situated (in the amino acid sequence) either before or after the cysteine residues that form the interchain disulfide bonds. COMP has been purified (Prochownik, E.V. *et al., J. Cell Biol. 109*:843-852 (1989)). Individuals affected with pseudoachondroplasia, who have considerably shortened stature as a result of premature cessation of bone growth, have been shown to have mutations in exon 17B of the COMP protein (*Nature Genetics 10*:325-329 (1995)).

In vitro assays have shown that platelet thrombospondin-1 is involved in thrombosis, fibrinolysis, wound healing, inflammation, tumor cell metastasis and angiogenesis. The major form of thrombospondin secreted by platelets and endothelial cells is TSP-1. Thrombospondin-1 (TSP-1) is an angiogenesis inhibitor that decreases tumor growth. Thrombospondin- 2 (TSP-2) is a related glycoprotein of similar structure and properties.

The thrombospondin type 1 repeats (TSRs; also "repeat regions" herein) have been shown to inhibit angiogenesis and HIV infection. However, other portions of the proteins have been shown to have a positive effect on endothelial cell

growth. Thromobospondin-1 and -2 are similar in terms of their molecular architecture. Thrombospondin-1and thrombospondin-2 each have three copies of the TSR. TSP-1 and TSP-2 are trimeric molecules. Thus, each fully assembled protein contains nine TSRs.

Whereas TSP-1 and TSP-2 are antiangiogenic, these proteins contain other domains that have additional activities that diminish the antiangiogenic activity. The isolated TSRs are more potent inhibitors of angiogenesis than the native molecules.

The ingrowth of new capillary networks into developing tumors is essential for the progression of cancer. Thus, the development of pharmaceuticals that inhibit the process of angiogenesis is an important therapeutic goal. As pointed out in a review by Folkman (Folkman, J., *Proc. Natl. Acad. Sci. USA 95*: 9064-9066, 1998), antiangiogenic therapy has little toxicity, does not require the therapeutic agent to enter tumor cells or cross the blood-brain barrier, controls tumor growth independently of growth of tumor cell heterogeneity, and does not induce drug resistance.

SUMMARY OF THE INVENTION

The invention includes chimeric proteins comprising: (1) a chimeric protein comprising the second and third type 1 repeats of human TSP-1, and which may also comprise the procollagen homology region of TSP-1; (2) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1; (3) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1; (4) a chimeric protein comprising the multimerization domain of human COMP, the procollagen region, and the first, second, and third type 1 repeats of human TSP-1; (5) a chimeric protein comprising the three type 1 repeats of human TSP-2, and which may also comprise the procollagen homology region of TSP-2; (6) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP,

and the three type 1 repeats of human TSP-2; and (7) variants of any of the above having anti-angiogenic activity. The invention further includes isolated nucleic acids encoding any of the above chimeric proteins, vectors comprising these nucleic acids, and host cells comprising any of said vectors. The chimeric proteins can be produced in host cells and used in methods for the treatment of a disease or medical condition characterized by abnormal or undesirable proliferation of blood vessels, such as that occurring in tumor growth.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a representation of the amino acid sequence of human TSP-1 (SEQ ID NO: 1). The type 1 repeats of TSP-1 are, as illustrated here, 1) amino acids 361-416; 2) amino acids 417-473; and 3) amino acids 474-530.

Figure 2 is a representation of the amino acid sequence of human TSP-2 (SEQ ID NO: 2). The type 1 repeats of TSP-2 are, as illustrated here, 1) amino acids 381-436; 2) amino acids 437-493; and 3) amino acids 494-550.

Figure 3 is a representation of the amino acid sequence of human COMP (SEQ ID NO: 3). The type 2 repeats of COMP are, as illustrated here, 1) amino acids 89-128; 2) amino acids 129-181; 3) amino acids 182-226; and 4) amino acids 227-268

Figures 4A and 4B together are a representation of the DNA sequence (SEQ ID NO: 4) of gene encoding a human COMP/TSP-1 chimeric protein and the amino acid sequence (SEQ ID NO: 5) of a human COMP/TSP-1 chimeric protein encoded by the DNA sequence above it.

Figure 5A and 5B together are a representation of the DNA sequence (SEQ ID NO: 6) of a gene encoding a human COMP/TSP-2 chimeric protein and the amino acid sequence (SEQ ID NO: 7) of a human COMP/TSP-2 chimeric protein encoded by the DNA sequence above it.

Figure 6 is a schematic representation of a few of the chimeric protein embodiments of the invention.

Figure 7 is a graph showing tumor volume (mm^3) at 7, 14 and 21 days in the experiment described in Example 3, in which mice were injected with an unaltered

(control) vector, pNeo (filled diamonds) or with an expression vector encoding COMP/TSP-1 chimeric protein (filled squares).

DETAILED DESCRIPTION OF THE INVENTION

Described herein is a protein that has the functional activity of the TSR but not other activities associated with TSP-1 or TSP-2, and is assembled into a multimeric structure. One embodiment of the invention is a chimeric protein that comprises the TSRs from TSP-1 or TSP-2 and the multimer assembly region of human cartilage oligomeric matrix protein (COMP), using a portion of the amino-terminal end. Other portions of TSP-1 or TSP-2 can be incorporated into the chimeric protein, such as the procollagen homology region of TSP-1 and/or TSP-2. The last two TSRs of TSP-1 are preferably used because the first TSR has the ability to activate transforming growth factor β (TGF-β), which stimulates tumor growth. The COMP assembly domain spontaneously forms a 5-stranded α-helical domain, allowing for the use of the COMP domain as a tool for pentamerization.

Thus, the COMP/TSP-1 construct contains the region for multimerization, the first type 2 repeat of human COMP (construct encodes amino acids 1-128) and the second and third TSRs of human TSP-1 (construct encodes amino acids 417-530). See the Table for active sequences of TSP-1 (taken from chapter 2, "The Primary Structure of the Thrombospondins" In *The Thrombospondin Gene Family* (J.C. Adams *et al.*, eds.) Springer-Verlag, Heidelberg (1995)). The assembled protein is a pentamer containing 10 copies of the TSR. Thus, COMP/TSP-1 and COMP/TSP-2 are expected to be more active than TSP-1 and TSP-2. COMP/TSP-1 and COMP/TSP-2 are expected to be correctly folded and multimeric so that they better mimic the natural proteins than peptides that are based on the TSR sequence.

The first type 2 repeat of COMP includes amino acid residues 73-130, based on the genomic sequence. The amount of COMP sequence at the 3' end can be increased or decreased to maximize activity. For example, two or more type 2 repeats of COMP can be included if moving the type 1 repeats of TSP-1 or TSP-2 farther out on the arms of the expressed protein increases its activity. Alternatively, "spacer" sequence not naturally occurring in COMP or in TSP-1 or TSP-2 can be

added. The COMP/TSP-2 construct contains the same region of COMP and the three TSRs of human TSP-2 (construct encodes amino acids 381-550). When it is assembled to a pentamer this chimeric protein will contain 15 TSRs. Because these proteins are derived from portions of human proteins, they should not be immunogenic in humans.

Table: Active Regions of Interest Within Thrombospondin-1

Domain	Sequence	Function
Procollagen homology	NGVQYRN (SEQ ID NO: 8)	Anti-angiogenesis
Type 1 repeats	CSVTCG (SEQ ID NO: 9)	Cell binding
	WSXWSXW (SEQ ID NO: 10)	Heparin binding
	GGWSHW (SEQ ID NO: 11)	TGF-β and Fibronectin binding
	RFK	TGF-β activation
	SPWDICSVTCGGGVQKRSR (SEQ ID NO: 12)	Anti-angiogenesis
Type 2 repeats	DVDEC$(X)_6$C$(X)_8$CENTDPGYNCLPC (SEQ ID NO: 13)	Calcium binding

In one aspect, the invention comprises polynucleotides or nucleic acid molecules that encode chimeric proteins having portions whose amino acid sequences are derived from human TSP-1. By the genomic structure, the type 1 repeats of TSP-1 are amino acid residues 359-414 (first), amino acid residues 415-473 (second), and 474-531 (third). In one case, the chimeric protein encoded by the polynucleotides of the invention comprises the second and third type 1 repeats of human TSP-1. Such a chimeric protein may also comprise the procollagen homology region and the first type 1 repeat of TSP-1. If amino acid sequences that activate TGF-β are included in the product protein, and are found to reduce anti-angiogenic activity, the RFK sequence can be mutated (to QFK, for example) to a

sequence that does not activate TGF-β, by appropriate manipulations of the nucleic acid molecule or construct encoding the chimeric proteins. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity (for example, plus or minus one order of magnitude in an assay) to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 4A and 4B. In another case, the chimeric proteins encoded by polynucleotides of the invention comprise the second and third type 1 repeats of human TSP-1, the multimerization domain of human COMP, and the first type 2 repeat of human COMP. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 4A and 4B.

In one aspect, the invention comprises polynucleotides or nucleic acid molecules that encode chimeric proteins having portions whose amino acid sequences are derived from human TSP-2. The genomic structure of the human TSP-2 gene, which would provide one way to define the boundaries of the repeats, has not been determined. In one case, the chimeric protein encoded by the polynucleotides of the invention comprises the three type 1 repeats of human TSP-2. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric proteins which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 5A and 5B. In another case, the chimeric protein encoded by polynucleotides of the invention comprises the three type 1 repeats of human TSP-2, and the multimerization domain of human COMP. In another case, the chimeric proteins encoded by the polynucleotides of the invention are variants of the immediately aforementioned chimeric protein which have activity that is similar in quality and quantity to the anti-angiogenic activity of the protein whose amino acid sequence is represented in Figures 5A and 5B.

The polynucleotides of the invention can be made by recombinant methods, can be made synthetically, can be replicated by enzymes in *in vitro* (e.g., PCR) or *in vivo* systems (e.g., by suitable host cells, when inserted into a vector appropriate for replication within the host cells), or can be made by a combination of methods. The polynucleotides of the invention can include DNA and its RNA counterpart.

As used herein, "nucleic acid," "nucleic acid molecule," "oligonucleotide" and "polynucleotide" include DNA and RNA and chemical derivatives thereof, including phosphorothioate derivatives and RNA and DNA molecules having a radioactive isotope or a chemical adduct such as a fluorophore, chromophore or biotin (which can be referred to as a "label"). The RNA counterpart of a DNA is a polymer of ribonucleotide units, wherein the nucleotide sequence can be depicted as having the base U (uracil) at sites within a molecule where DNA has the base T (thymidine).

Isolated nucleic acid molecules or polynucleotides can be purified from a natural source or can be made recombinantly. Polynucleotides referred to herein as "isolated" are polynucleotides purified to a state beyond that in which they exist in cells. They include polynucleotides obtained by methods described herein, similar methods or other suitable methods, and also include essentially pure polynucleotides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant polynucleotides that have been isolated. The term "isolated" as used herein for nucleic acid molecules, indicates that the molecule in question exists in a physical milieu distinct from that in which it occurs in nature. For example, an isolated polynucleotide may be substantially isolated with respect to the complex cellular milieu in which it naturally occurs, and may even be purified essentially to homogeneity, for example as determined by agarose or polyacrylamide gel electorphoresis or by A_{260}/A_{280} measurements, but may also have further cofactors or molecular stabilizers (for instance, buffers or salts) added.

The invention further comprises the polypeptides encoded by the isolated nucleic acid molecules of the invention. Thus, for example, the invention relates to fusion proteins, comprising a portion of TSP-1 which comprises the second and third type 1 repeats, linked to a second moiety not occurring in TSP-1 as found in

nature. In an analogous manner, the invention relates also to fusion proteins, comprising TSP-2 or a functional portion thereof such as one or more repeat regions as a first moiety, linked to second moiety not occurring in TSP-2 as found in nature. The second moiety can be an amino acid, peptide or polypeptide, and can have enzymatic or binding activity of its own. The first moiety can be in an N-terminal location, C-terminal location or internal to the fusion protein. In one embodiment, the fusion protein comprises the portion of human TSP-1 described immediately above, or human TSP-2 or a portion thereof as the first moiety, and a second moiety comprising a linker sequence and an affinity ligand.

Another aspect of the invention relates to a method of producing a chimeric protein of the invention, or a variant thereof, and to expression systems and host cells containing a vector appropriate for expression of a chimeric protein of the invention. Variants of the chimeric protein include those having amino acid sequences that differ from those sequences in Figures 4A and 4B, and Figures 5A and 5B, wherein those variants have several, such as 5 to 10, 1 to 5, or 3, 2 or 1 amino acids substituted, deleted, or added, in any combination, compared to the sequences in Figures 4A and 4B and Figures 5A and 5B. In one embodiment, variants have silent substitutions, additions and deletions that do not alter the properties and activities of the chimeric protein. Variants can also be modified polypeptides in which one or more amino acid residues are modified, and mutants comprising one or more modified residues.

Proteins and polypeptides described herein can be assessed for their angiogenic activity by using an assay such as those described in Tolsma, S.S. *et al.*, *J. Cell Biol. 122(2)*:497-511 (1993), one which measures the migration of bovine adrenal capillary endothelial cells in culture, and one which tests migration of cells into a sponge containing an agent to be tested for activity. A further test for angiogenesis, which can also be adapted also to test anti-angiogenesis activity, is described in Polverini, P.J. *et al.*, *Methods. Enzymol. 198*:440-450 (1991).

Cells that express such a chimeric protein or a variant thereof can be made and maintained in culture, under conditions suitable for expression, to produce protein for isolation. These cells can be procaryotic or eucaryotic. Examples of

procaryotic cells that can be used for expression (as "host cells"; "cell" including herein cells of tissues, cell cultures, cell strains and cell lines) include *Escherichia coli, Bacillus subtilis* and other bacteria. Examples of eucaryotic cells that can be used for expression include yeasts such as *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Pichia pastoris* and other lower eucaryotic cells, and cells of higher eucaryotes such as those from insects and mammals. Suitable cells of mammalian origin include primary cells, and cell lines such as CHO, HeLa, 3T3, BHK, COS, 293, and Jurkat cells. Suitable cells of insect origin include primary cells, and cell lines such as SF9 and High five cells. (See, e.g., Ausubel, F.M. *et al.*, eds. *Current Protocols in Molecular Biology,* Greene Publishing Associates and John Wiley & Sons Inc., (containing Supplements up through 1998)).

In one embodiment, host cells that produce a recombinant chimeric protein, variant, or portions thereof can be made as follows. A gene encoding a chimeric protein described herein can be inserted into a nucleic acid vector, e.g., a DNA vector, such as a plasmid, virus or other suitable replicon (including vectors suitable for use in gene therapy, such as those derived from adenovirus or others; see, for example Xu, M. *et al., Molecular Genetics and Metabolism 63*:103-109, 1998) can be present in a single copy or multiple copies, or the gene can be integrated in a host cell chromosome. A suitable replicon or integrated gene can contain all or part of the coding sequence for the protein or variant, operably linked to one or more expression control regions whereby the coding sequence is under the control of transcription signals and linked to appropriate translation signals to permit translation. The vector can be introduced into cells by a method appropriate to the type of host cells (e.g., transformation, electroporation, infection). For expression from the gene, the host cells can be maintained under appropriate conditions (e.g., in the presence of inducer, normal growth conditions, etc.). Proteins or polypeptides thus produced can be recovered (e.g., from the cells, the periplasmic space, culture medium) using suitable techniques.

The invention also relates to isolated proteins or polypeptides encoded by nucleic acids of the present invention. Isolated proteins can be purified from a natural source or can be made recombinantly. Proteins or polypeptides referred to

herein as "isolated" are proteins or polypeptides purified to a state beyond that in which they exist in cells and include proteins or polypeptides obtained by methods described herein, similar methods or other suitable methods, and also include essentially pure proteins or polypeptides, proteins or polypeptides produced by chemical synthesis or by combinations of biological and chemical methods, and recombinant proteins or polypeptides which are isolated. Thus, the term "isolated" as used herein, indicates that the polypeptide in question exists in a physical milieu distinct from the cell in which its biosynthesis occurs. For example, an isolated COMP/TSP-1 or COMP/TSP-2 chimeric protein may be purified essentially to homogeneity, for example as determined by PAGE or column chromatography (for example, HPLC), but may also have further cofactors or molecular stabilizers added to the purified protein to enhance activity. In one embodiment, proteins or polypeptides are isolated to a state at least about 75% pure; more preferably at least about 85% pure, and still more preferably at least about 95% pure, as determined by Coomassie blue staining of proteins on SDS-polyacrylamide gels.

Chimeric or fusion proteins can be produced by a variety of methods. For example, a chimeric protein can be produced by the insertion of a TSP gene or portion thereof into a suitable expression vector, such as Bluescript SK +/- (Stratagene), pGEX-4T-2 (Pharmacia), pET-15b, pET-20b(+) or pET-24(+) (Novagen). The resulting construct can be introduced into a suitable host cell for expression. Upon expression, chimeric protein can be purified from a cell lysate by means of a suitable affinity matrix (see e.g., *Current Protocols in Molecular Biology* (Ausubel, F.M. *et al.*, eds., Vol. 2, pp. 16.4.1-16.7.8, containing supplements up through Supplement 44, 1998).

Polypeptides of the invention can be recovered and purified from cell cultures by well-known methods. The recombinant protein can be purified by ammonium sulfate precipitation, heparin-Sepharose affinity chromatography, gel filtration chromatography and/or sucrose gradient ultracentrifugation using standard techniques. Further methods that can be used for purification of the polypeptide include ethanol precipitation, acid extraction, anion or cation exchange chromatography, phosphocellulose chromatography, hydrophobic interaction

chromatography, affinity chromatography, hydroxylapatite chromatography and high performance liquid chromatography. Known methods for refolding protein can be used to regenerate active conformation if the polypeptide is denatured during isolation or purification.

The method to construct genes encoding COMP/TSP-1 or COMP/TSP-2 hybrid proteins can be applied more broadly to produce polynucleotides, and vectors and host cells comprising such polynucleotides, wherein the polynucleotides encode COMP/endostatin, COMP/angiostatin, COMP/platelet factor 4, or COMP/prolactin, for example. In each case, a portion of a polynucleotide known to encode full-length human endostatin, angiostatin, platelet factor 4 (GenBank Accession No. M25897) or prolactin (GenBank Accession No. V00566), can be chosen for cloning into a COMP cDNA as illustrated herein for COMP/TSP-1 and COMP/TSP-2 DNA constructs. Thus, the invention also includes COMP/endostatin, COMP/angiostatin, COMP/platelet factor 4, and COMP/prolactin chimeric proteins encoded by such nucleic acid constructs. See Figure 6 for a schematic representation of the structure of COMP/endostatin.

In addition, a portion of the endostatin, angiostatin, platelet factor 4 or prolactin coding regions, wherein that portion encodes a polypeptide having anti-angiogenic activity, can be added to or incorporated into a DNA construct encoding COMP/TSP-1, such that a TSP-1-derived polypeptide and a polypeptide derived from endostatin, angiostatin, platelet factor 4 or prolactin are produced fused together in tandem on the same "arm" of the "5-armed" COMP-multimerized pentamer. Different expression constructs can be introduced into the same host cells such that two or more chimeric protein "arms" of different types (e.g., COMP/angiostatin and COMP/TSP-1 or COMP/TSP-2) are joined at the COMP multimerization domain.

Chimeric protein antiangiogenic agents can be used, for example, after surgery or radiation to prevent recurrence of metastases, in combination with conventional chemotherapy, immunotherapy, or various types of gene therapy not necessarily directed against angiogenesis.

Construction of COMP/TSP-1P Expression Vectors

Expression vectors that can be used to produce COMP/TSP-1P, a chimeric protein that includes the procollagen homology region (see Figure 6), can be produced from two distinct cDNAs. The COMP portion is identical to that in the Examples described herein. For TSP-1, a new forward primer (GAT GAC GTC ACT GAA GAG AAC AAA GAG) (SEQ ID NO: 14) and the same reverse primer as described in the Examples can be used to produce a PCR product that is approximately 750 base pairs in size and has an AatII restriction endonuclease site at the 5' end and an XbaI restriction endonuclease site at the 3' end. The product codes for amino acids 284-530 and includes the procollagen homology region (exons 6 and 7) and type 1 repeats. If inclusion of the TGF-β activating sequence (RFK) that is in the first type 1 repeat is found to reduce the antitumor activity, this sequence will be mutated to an inactive sequence (QFK, for example) using an oligonucleotide-directed mutagenesis kit (Amersham). The COMP/TSP-1P expression vector can be constructed by cutting the PCR product with AatII and XbaI and cloning it into the COMP cDNA cut with the same enzymes. The protein can be expressed using the methods that have been described for COMP/TSP-1 and COMP/TSP-2.

Construction of COMP/Endostatin Expression Vectors

The strategy for making multimers of the TSP-1 and TSP-2 can be used to make multimers of other anti-angiogenic proteins. For example, if the active region of endostatin is prepared by PCR and cloned into the COMP cDNA, a pentameric structure of endostatin can be made when this construct is expressed (O'Reilly M.D., *et al., Cell 88*:277-285, (1997)). In addition, if the COMP/TSP-1 and the COMP/endostatin genes are expressed concurrently within the same cells, mixed pentamers of COMP/TSP-1 and COMP/endostatin subunits are made. The mixed multimer allows simultaneous treatment with the two reagents by delivery of a single therapeutic. An additive or synergistic effect of the two agents may significantly increase the efficacy of this reagent as compared to that of each reagent alone. For example, combination therapy with angiostatin and endostatin has eradicated tumors in mice (Boehm, T. *et al., Nature 390*:404-407, 1997).

The cDNA for endostatin can be prepared by PCR of liver cDNA or from an isolated cDNA clone for collagen XVIII (GenBank accession no. L22548). The human endostatin cDNA can be produced by PCR with the forward primer GAT GAC GTC CAC AGC CAC CGC G (SEQ ID NO: 15) and the reverse primer GAT TCT AGA CTA CTT GGA GGC AGT CAT G (SEQ ID NO: 16). The resulting PCR product is approximately 560 base pairs and encodes amino acids 1 to 184 of human endostatin (Sasaki, T., *et al., EMBO J.*, *17*:4249-4256, 1998). The COMP/endostatin expression vector can be constructed by cutting the PCR product with AatII and XbaI, and cloning it into cDNA cut with the same enzymes. The protein can be expressed using the methods that have been described herein for COMP/TSP-1 and COMP/TSP-2. Angiostatin, as it was isolated from mice bearing Lewis lung carcinoma, includes the first four kringle domains of plasminogen (amino acids 98-440) (O'Reilly, M.S., *et al., Cell 79*:315-328, 1994). It should be noted that smaller constructs that contain fewer kringle domains should also be active based on published data (Griscelli, F., *et al., Proc. Natl. Acad. Sci. USA 95*:6367-6372, 1998). A 16,000 dalton fragment of prolactin and platelet factor 4 have also been reported to inhibit angiogenesis (Clapp, C. *et al., Endocrinology 133*:1292-1299, 1993; Gapta, S.K., *et al., Proc. Natl. Acad. Sci. USA 92*:7799-7803, 1995).

Also included in the inventions are compositions containing, as a biological ingredient, an anti-angiogenic chimeric protein, or a variant thereof to inhibit angiogenesis in mammalian tissues, and use of such compositions in the treatment of diseases and conditions characterized by, or associated with, angiogenic activity. Such methods can involve administration by oral, topical, injection, implantation, sustained release, or other delivery methods that bring one or more anti-angiogenic chimeric proteins in contact with cells whose growth is to be inhibited.

The present invention includes a method of treating an angiogenesis-mediated disease with a therapeutically effective amount of one or more anti-angiogenic chimeric proteins. Angiogenesis-mediated diseases can include, but are not limited to, cancers, solid tumors, tumor metastasis, benign tumors (*e.g.*, hemangiomas, acoustic neuromas, neurofibromas, trachomas, and pyogenic

granulomas), rheumatoid arthritis, psoriasis, ocular angiogenic diseases (*e.g.*, diabetic retinopathy, retinopathy of prematurity, macular degeneration, corneal graft rejection, neovascular glaucoma, retrolental fibroplasia, rubeosis), Osler-Webber Syndrome, myocardial angiogenesis, plaque neovascularization, telangiectasia, hemophiliac joints, angiofibroma, and wound granulation.

"Cancer" means neoplastic growth, hyperplastic or proliferative growth or a pathological state of abnormal cellular development and includes solid tumors, non-solid tumors, and any abnormal cellular proliferation, such as that seen in leukemia. As used herein, "cancer" also means angiogenesis-dependent cancers and tumors, *i.e.*, tumors that require for their growth (expansion in volume and/or mass) an increase in the number and density of the blood vessels supplying them with blood. "Regression" refers to the reduction of tumor mass and size. As used herein, the term "therapeutically effective amount" means the total amount of each active component of the composition or method that is sufficient to show a meaningful benefit to a treated human or other mammal, *i.e.*, treatment, healing, prevention or amelioration of the relevant medical condition, or an increase in rate of treatment, healing, prevention or amelioration of such conditions. More specifically, for example, a therapeutically effective amount of an anti-angiogenic chimeric protein can cause a measurable reduction in the size or numbers of tumors, or in their rate of growth or multiplication, compared to untreated tumors. Other methods of assessing a "therapeutically effective amount," can include the result that blood vessel formation is measurably reduced in treated tissues compared to untreated tissues.

One or more anti-angiogenic chimeric proteins may be used in combination with other compositions and procedures for the treatment of diseases. For example, a tumor may be treated conventionally with surgery, radiation, chemotherapy, or immunotherapy, combined with anti-angiogenic chimeric proteins, and then anti-angiogenic chimeric proteins may be subsequently administered to the patient to extend the dormancy of micrometastases and to stabilize and inhibit the growth of any residual primary tumor.

The compositions may further contain other agents which either enhance the activity of the protein or compliment its activity or use in treatment, such as

chemotherapeutic or radioactive agents. Such additional factors and/or agents may be included in the composition to produce a synergistic effect with protein of the invention, or to minimize side effects. Additionally, administration of the composition of the present invention may be administered concurrently with other therapies, *e.g.*, administered in conjunction with a chemotherapy, immunotherapy or radiation therapy regimen.

The angiogenesis-modulating composition of the present invention may be a solid, liquid or aerosol and may be administered by any known route of administration. Examples of solid compositions include pills, creams, and implantable dosage units. The pills may be administered orally, the therapeutic creams may be administered topically. The implantable dosage unit may be administered locally, for example at a tumor site, or may be implanted for systemic release of the angiogenesis-modulating composition, for example subcutaneously. Examples of liquid composition include formulations adapted for injection subcutaneously, intravenously, intraarterially, and formulations for topical and intraocular administration. Examples of aerosol formulation include inhaler formulation for administration to the lungs.

The anti-angiogenic chimeric proteins can be provided as isolated and substantially purified proteins in pharmaceutically acceptable formulations (including aqueous or nonaqueous carriers or solvents) using formulation methods known to those of ordinary skill in the art. These formulations can be administered by standard routes. In general, the combinations may be administered by the topical, transdermal, intraperitoneal, intracranial, intracerebroventricular, intracerebral, intravaginal, intrauterine, oral, rectal or parenteral (*e.g.*, intravenous, intraspinal, subcutaneous or intramuscular) route. In addition, the anti-angiogenic chimeric proteins may be incorporated into biodegradable polymers allowing for sustained release of the compound, the polymers being implanted in the vicinity of where drug delivery is desired, for example, at the site of a tumor, or implanted so that the anti-angiogenic chimeric proteins is slowly released systemically. Osmotic minipumps may also be used to provide controlled delivery of high concentrations of anti-angiogenic chimeric proteins through cannulae to the site of interest, such as directly

into a growth or into the vascular supply to that growth. The biodegradable polymers and their use are described, for example, in detail in Brem *et al.* (1991) (*J. Neurosurg.* 74:441-446), which is hereby incorporated by reference in its entirety.

As used herein, the terms "pharmaceutically acceptable," as it refers to compositions, carriers, diluents and reagents, represents that the materials are capable of administration to or upon a mammal with a minimum of undesirable physiological effects such as nausea, dizziness, gastric upset and the like. The preparation of a pharmacological composition that contains active ingredients dissolved or dispersed therein is well understood in the art and need not be limited based on formulation. Typically, such compositions are prepared as injectables either as liquid solutions or suspensions, however, solid forms suitable for solution, or suspensions, in liquid prior to use can also be prepared. The preparation can also be emulsified, for example, in liposomes.

The dosage of the anti-angiogenic chimeric proteins of the present invention will depend on the disease state or condition being treated and other clinical factors such as weight and condition of the human or animal and the route of administration of the compound. It is to be understood that the present invention has application for both human and veterinary use. The methods of the present invention contemplate single as well as multiple administrations, given either simultaneously or over an extended period of time.

The present invention also encompasses gene therapy whereby a polynucleotide encoding one or more anti-angiogenic chimeric proteins or one or more variants thereof, is introduced and regulated in a patient. Various methods of transferring or delivering DNA to cells for expression of the gene product protein, otherwise referred to as gene therapy, are disclosed in *Gene Transfer into Mammalian Somatic Cells in Vivo*, N. Yang (1992) *Crit. Rev. Biotechnol.* 12(4):335-356, which is hereby incorporated by reference. Gene therapy encompasses incorporation of DNA sequences into somatic cells or germ line cells for use in either ex vivo or in vivo therapy. Gene therapy can function to replace genes, augment normal or abnormal gene function, and to combat infectious diseases and other pathologies.

Strategies for treating these medical problems with gene therapy include therapeutic strategies such as identifying the defective gene and then adding a functional gene to either replace the function of the defective gene or to augment a slightly functional gene; or prophylactic strategies, such as adding a gene for the product protein that will treat the condition or that will make the tissue or organ more susceptible to a treatment regimen. For example, a gene encoding an anti-angiogenic chimeric protein may be inserted into tumor cells of a patient and thus inhibit angiogenesis.

Gene transfer methods for gene therapy fall into three broad categories: physical (*e.g.*, electroporation, direct gene transfer and particle bombardment), chemical (*e.g.*, lipid-based carriers, or other non-viral vectors) and biological (*e.g.*, virus-derived vector and receptor uptake). For example, non-viral vectors may be used which include liposomes coated with DNA. Such liposome/DNA complexes may be directly injected intravenously into the patient. It is believed that the liposome/DNA complexes are concentrated in the liver where they deliver the DNA to macrophages and Kupffer cells. These cells are long lived and thus provide long term expression of the delivered DNA. Additionally, vectors or the "naked" DNA of the gene may be directly injected into the desired organ, tissue or tumor for targeted delivery of the therapeutic DNA.

In vivo gene transfer involves introducing the DNA into the cells of the patient when the cells are within the patient. Methods include using virally mediated gene transfer using a noninfectious virus to deliver the gene in the patient or injecting naked DNA into a site in the patient and the DNA is taken up by a percentage of cells in which the gene product protein is expressed. Additionally, the other methods described herein, such as use of a "gene gun," may be used for in vitro insertion of anti-angiogenic chimeric proteins DNA or anti-angiogenic chimeric proteins regulatory sequences.

Chemical methods of gene therapy may involve a lipid based compound, not necessarily a liposome, to transfer the DNA across the cell membrane. Lipofectins or cytofectins, lipid-based positive ions that bind to negatively charged DNA, make a complex that can cross the cell membrane and provide the DNA into the interior of

the cell. Another chemical method uses receptor-based endocytosis, which involves binding a specific ligand to a cell surface receptor and enveloping and transporting it across the cell membrane. The ligand binds to the DNA and the whole complex is transported into the cell. The ligand gene complex is injected into the blood stream and then target cells that have the receptor will specifically bind the ligand and transport the ligand-DNA complex into the cell.

Many gene therapy methodologies employ viral vectors to insert genes into cells. For example, altered retrovirus vectors have been used in *ex vivo* methods to introduce genes into peripheral and tumor-infiltrating lymphocytes, hepatocytes, epidermal cells, myocytes, or other somatic cells. These altered cells are then introduced into the patient to provide the gene product from the inserted DNA.

Viral vectors have also been used to insert genes into cells using in vivo protocols. To direct the tissue-specific expression of foreign genes, cis-acting regulatory elements or promoters that are known to be tissue-specific can be used. Alternatively, this can be achieved using *in situ* delivery of DNA or viral vectors to specific anatomical sites *in vivo*. For example, gene transfer to blood vessels *in vivo* was achieved by implanting *in vitro* transduced endothelial cells in chosen sites on arterial walls. The virus infected surrounding cells which also expressed the gene product. A viral vector can be delivered directly to the *in vivo* site, by a catheter for example, thus allowing only certain areas to be infected by the virus, and providing long-term, site specific gene expression. *In vivo* gene transfer using retrovirus vectors has also been demonstrated in mammary tissue and hepatic tissue by injection of the altered virus into blood vessels leading to the organs.

Viral vectors that have been used for gene therapy protocols include but are not limited to, retroviruses, other RNA viruses such as poliovirus or Sindbis virus, adenovirus, adeno-associated virus, herpes viruses, SV40, vaccinia and other DNA viruses. Replication-defective murine retroviral vectors have been widely utilized gene transfer vectors.

Carrier mediated gene transfer *in vivo* can be used to transfect foreign DNA into cells. The carrier-DNA complex can be conveniently introduced into body fluids or the bloodstream and then site-specifically directed to the target organ or

tissue in the body. Both liposomes and polycations, such as polylysine, lipofectins or cytofectins, can be used. Liposomes can be developed which are cell specific or organ specific and thus the foreign DNA carried by the liposome will be taken up by target cells. Injection of immunoliposomes that are targeted to a specific receptor on certain cells can be used as a convenient method of inserting the DNA into the cells bearing the receptor. Another carrier system that has been used is the asialoglycoprotein/polylysine conjugate system for carrying DNA to hepatocytes for *in vivo* gene transfer.

The gene therapy protocol for transfecting anti-angiogenic chimeric proteins into a patient may either be through integration of a gene encoding an anti-angiogenic chimeric protein into the genome of the cells, into minichromosomes or as a separate replicating or non-replicating DNA construct in the cytoplasm or nucleoplasm of the cell. Anti-angiogenic chimeric proteins expression may continue for a long-period of time or may be reinjected periodically to maintain a desired level of the anti-angiogenic chimeric proteins protein in the cell, the tissue or organ or a determined blood level.

EXAMPLES

Example 1: Construction of COMP/TSP-1 and COMP/TSP-2

The chimeric expression vectors have been produced from three distinct cDNAs. The first is a clone for human cartilage oligomeric matrix protein (COMP) and was isolated from a λgtll chondrocyte cDNA library (Doege, K.J, *et al., J. Biol. Chem. 266:*894-902 (1991)). This is an almost full-length clone for the COMP mRNA that only lacks a small region of the 5'-untranslated region. This clone (hCOMP-95) was used previously to determine the sequence of human COMP (GenBank Accession No. L32137; *Genomics, 24*:435-439 (1994)).

The second cDNA was produced using the polymerase chain reaction (PCR) with the human thrombospondin-1 (TSP-1) gene as the template. The TSP-1 clones were isolated from a human endothelial cell library (*J. Cell Biol. 103*:1635-1648

(1986)). The forward primer (GAT GAC GTC GAT GGT GGC TGG AGC CAC) (SEQ ID NO: 17) and the reverse primer (GAT CTA GAT TGG ACA GTC CTG CTT G) (SEQ ID NO: 18) produce a PCR product that is approximately 354 basepairs in size and has an Aat II restriction endonuclease site at the 5' end and an Xba I restriction endonuclease site at the 3' end. The PCR product encodes amino acids 417 to 530 and includes the second and third type 1 repeats of TSP-1 (see Figure 1 for the numbering of amino acids in TSP-1). The coding sequence for the first type 1 repeat was not included in the PCR product, by design, because it contains an RFK sequence that has been shown to activate TGF-β. This activity is not required to inhibit angiogenesis and it may produce unwanted secondary effects on numerous cell types. Vectors that include the first type 1 repeat can be constructed, using the same approach, if this region is found to enhance the antiangiogenic activity or other activities.

The third cDNA was produced by PCR with a human heart cDNA library (catalog no. 936208 from Stratagene, LaJolla, CA) as the template. The forward primer (GAT GAC GTC GAG GAG GGC TGG TCT CCG) (SEQ ID NO: 19) and the reverse primer (GAT CTA GAC ACG GGG CAG CTC CTC TTG) (SEQ ID NO: 20) produced a PCR product that is approximately 520 base pairs in size and has an Aat II restriction endonuclease site at the 5' end and an Xba I restriction endonuclease site at the 3' end. The PCR product codes for amino acids 381 to 550 of TSP-2 and, includes all three type 1 repeats of TSP-2 (see Figure 2 for numbering of amino acids in TSP-2). The sequence of the PCR primers was based on the human TSP-2 sequence in the GenBank database (Accession No. L12350). The sequences of the PCR products were determined to establish that mutations that affect the amino acid sequence had not been introduced during the PCR.

The COMP/TSP-1 and COMP-TSP-2 expression vectors were constructed by cutting the PCR products with Aat II and Xba I and subcloning them into the COMP cDNA vector [derived from Bluescript (Stratagene, La Jolla, CA)] cut with the same enzymes. The portion of COMP that was retained includes the signal sequence, the regions required for pentamerization and the first type 2 repeat (amino acids 1 to 128 on the enclosed sequence; Figure 3). Since there was an internal Aat

II site in the TSP-2 PCR product, it had to be cloned into the vector in two steps. A 430 basepair Aat II/Xba I fragment of the TSP-2 PCR product was subcloned into the vector containing the portion of COMP as a first step. The resulting subclone was cut with Aat II, and a 90 base pair Aat II fragment of the PCR product was ligated into the expression vector. The final forms of the cDNAs were confirmed to have the predicted structure by nucleotide sequencing. They were then cut with Eco R1 and Xba I and ligated into the pcDNA 3.1 (Invitrogen; Carlsbad, CA) vector cut with the same enzymes. The DNA sequences of COMP/TSP-1 and COMP/TSP-2 are shown in Figures 4A and 4B and Figures 5A and 5B, respectively. The predicted molecular weights of the subunits of COMP/TSP-1 and COMP/TSP-2 should be approximately 24,200 and 30,000, respectively. The fully assembled COMP/TSP-1 and COMP/TSP-2 proteins should be 121,000 Da and 150,000 Da, respectively. The amino acid sequences of these proteins are shown in Figures 4A and 4B and Figures 5A and 5B, respectively.

Example 2: Production of Isolated COMP/TSP-1 and COMP/TSP-2

To express these chimeric proteins, the expression vectors can be transfected into human kidney 293 cells using the Lipofectin protocol (Gibco Laboratories). The cells can be selected with Zeocin and individual clones can be grown. The secretion of COMP/TSP-1 and COMP/TSP-2 can be monitored with western blotting using polyclonal antibodies to the region of COMP that is present in both expressed proteins. These antibodies have been produced by immunizing rabbits with a synthetically produced peptide, having an amino acid sequence derived from the N-terminal end of COMP, linked to a carrier protein. The amino acid sequence of the peptide is: SDLGPQMLRELQETN (SEQ ID NO: 21). A clone that expresses high levels of the protein can be grown in large volume flasks and in serum free media.

Example 3: Inhibition of Tumor Growth by COMP/TSP-1

A cDNA of thrombospondin-1 (TSP-1) containing the second and third type-1 repeats and the COMP assembly sequence (COMP/TSP-1) was produced by PCR

using constructs derived as above as template, and was cloned into the expression vector pNeo (Invitrogen, Carlsbad, CA). Both the resulting COMP/TSP-1 construct and the unaltered vector alone were transfected into the human squamous carcinoma cell line A431 (Streit, M., *et al., American Journal of Pathology 155*:441-452, 1999), and positive clones were selected using Geneticin at a concentration of 800 μg/ml. The growth curves of positive clones were determined over an 8 day period. Clones of pNeo- and COMP/TSP-1 construct-transfected cells that had similar growth curves were selected to test the effect of the chimeric protein on tumor growth in nude mice. A total of five mice pre group were injected intradermally at the shoulders with 5×10^6 cells per site, two sites per mouse. Every week the tumors were measured with calipers. At three weeks, the mice were sacrificed and the tumors were removed for further studies. As can be seen from Figure 7, expression of COMP/TSP-1 caused inhibition of the growth of the tumors in this model.

All references (e.g., journal articles, books, published patent applications and patents, etc.) cited herein are hereby incorporated by reference.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A nucleic acid molecule encoding a chimeric protein comprising the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

2. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

3. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

4. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1.

5. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

6. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human endostatin, wherein the chimeric protein has anti-angiogenic activity.

7. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human angiostatin, wherein the chimeric protein has anti-angiogenic activity.

8. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human prolactin, wherein the chimeric protein has anti-angiogenic activity.

9. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP and a portion of human platelet factor 4, wherein the chimeric protein has anti-angiogenic activity.

10. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region, and the first, second, and third type 1 repeats of human TSP-1.

11. A nucleic acid molecule encoding a protein having the amino acid sequence SEQ ID NO: 5.

12. A vector comprising nucleic acid encoding a chimeric protein comprising the second and third type 1 repeats of human TSP-1 but not the TGF-β activation region of human TSP-1.

13. A host cell comprising the vector of Claim 12.

14. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

15. A host cell comprising the vector of Claim 14.

16. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, said method comprising maintaining the host cell of Claim 15 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

17. The method of Claim 16 further comprising isolating the chimeric protein.

18. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

19. A host cell comprising the vector of Claim 18.

20. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1, said method comprising maintaining the host cell of Claim 19 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

21. The method of Claim 20 further comprising isolating the chimeric protein.

22. A vector comprising nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region, and the first, second, and third type 1 repeats of human TSP-1.

23. A vector comprising nucleic acid encoding a protein having the amino acid sequence SEQ ID NO: 5.

24. A host cell comprising the vector of Claim 23.

25. A chimeric protein comprising the second and third type 1 repeat of human TSP-1, but not the TGF-β activation region of human TSP-1.

26. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1.

27. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1.

28. A chimeric protein comprising the multimerization domain of human COMP, the procollagen homology region of TSP-1, and the first, second, and third type 1 repeats of human TSP-1.

29. A chimeric protein comprising the multimerization domain of human COMP and a portion of human endostatin, wherein the chimeric protein has anti-angiogenic activity.

30. A chimeric protein comprising the multimerization domain of human COMP and a portion of human angiostatin, wherein the chimeric protein has anti-angiogenic activity.

31. A chimeric protein comprising the multimerization domain of human COMP and a portion of human prolactin, wherein the chimeric protein has anti-angiogenic activity.

32. A chimeric protein comprising the multimerization domain of human COMP and a portion of human platelet factor 4, wherein the chimeric protein has anti-angiogenic activity.

33. A protein having the amino acid sequence SEQ ID NO: 5.

34. An isolated nucleic acid molecule encoding a chimeric protein comprising the three type 1 repeats of human TSP-2.

35. A vector comprising nucleic acid encoding a chimeric protein comprising the three type 1 repeats of human TSP-2.

36. A host cell comprising the vector of Claim 35.

37. A method for producing a chimeric protein which comprises the three type 1 repeats of human TSP-2, said method comprising maintaining the host cell of Claim 36 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

38. The method of Claim 37 further comprising isolating the chimeric protein.

39. A nucleic acid molecule encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

40. A vector comprising isolated nucleic acid encoding a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

41. A host cell comprising the vector of Claim 40.

42. A method for producing a chimeric protein which comprises the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2, said method comprising maintaining the host cell of Claim 41 under conditions suitable for expression of said nucleic acid, whereby said protein is produced.

43. The method of Claim 42 further comprising isolating the chimeric protein.

44. A nucleic acid molecule encoding a protein having the amino acid sequence SEQ ID NO: 7.

45. A vector comprising nucleic acid encoding a protein having the amino acid sequence SEQ ID NO: 7.

46. A host cell comprising the vector of Claim 45.

47. A chimeric protein comprising the three type 1 repeats of human TSP-2.

48. A chimeric protein comprising the procollagen homology region of TSP-2 and the three type 1 repeats of human TSP-2.

49. A chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

50. A protein having the amino acid sequence SEQ ID NO: 7.

51. A method for inhibiting angiogenesis in a human or other mammal, the method comprising administering to the human or other mammal a therapeutically effective amount of an anti-angiogenic chimeric protein.

52. The method of Claim 51 wherein the anti-angiogenic chimeric protein is selected from the group consisting of:

a) a chimeric protein comprising the second and third type 1 repeats of human TSP-1;

b) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1;

c) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the second and third type 1 repeats of human TSP-1, but not the TGF-β activation region of human TSP-1;

d) a chimeric protein comprising the multimerization domain of human COMP, the procollagen region, and the first, second, and third type 1 repeats of human TSP-1; and

e) a chimeric protein comprising the three type 1 repeats of human TSP-2; and (6) a chimeric protein comprising the multimerization domain of human COMP, the first type 2 repeat of human COMP, and the three type 1 repeats of human TSP-2.

53. The method of Claim 51 wherein the anti-angiogenic protein is administered locally at the site of one or more growths.

human thrombospondin-1

NH2	1	NRIPESGGDNSVFDIFELTGAARKGSGRRLVKGPDPSSPAFRIEDANLIPPVPDDKFQDL
	61	VDAVRTEKGFLLLASLRQMKKTRGTLLALERKDHSGQVFSVVSNGKAGTLDLSLTVQGKQ
	121	HVVSVEEALLATGQWKSITLFVQEDRAQLYIDCEKMENAELDVPIQSVFTRDLASIARLR
	181	IAKGGVNDNFQGVLQNVRFVFGTTPEDILRNKGCSSSTSVLLTLDNNVVNGSSPAIRTNY
	241	IGHKTKDLQAICGISCDELSSM
P	263	VLELRGLRTIVTTLQDSIRKVTEENKELANELRRPPLCYHNGVQYRNNE
	312	EWTVDSCTECHCQNSVTICKKVSCPIMPCSNATVPDGECCPRCWPSDSA
type 1	361	DDGWSPWSEWTSCSTSCGNGIQQRGRSCDSLNNR-----CEGSSVQTRTCHIQECDKRFKQ
	417	DGGWSHWSPWSS/CSVTCGDGVITRIRLCNSPSPQMNGKPCEGEARETKACKKDACPI
	474	NGGWGPWSPWDICSVTCGGGVQKRSRLCNNPTPQFGGKDCVGDVTENQICNKQDCPI
type 2	531	DGCLSNP--CFAGV---KCT---SYPDGSWKCGACPPGYSG-------------NGIQCTDV
	572	DECKEVPDACFNHNGEHRCEN----TDPGYNCLPCPPRFTGSQPFGQGVEHATANKQVCKPR
	630	NPCTDGTHDCNKNA---KCNYLGHYSDPMYRC-ECKPGYAG-------------NGIICGE
	674	DTDLDGWPNENLVCVANATYHCKK
type 3	698	DNCPNLPNSGQEDYDKDGIGDACDD--DDDNDKIPDDR
	734	DNCPFHYNPAQYDYDRDDVGDRC
	757	DNCPYNHNPDQADTDNNGEGDACAA--DIDGDGILNER
	793	DNCQYVYNVDQRDTDMDGVGDQC
	816	DNCPLEHNPDQLDSDSDRIGDTCDNNQDIDEDGHQNNL
	854	DNCPYVPNANQADHDKDGKGDACDH--DDDNDGIPDDK
	890	DNCRLVPNPDQKDSDGDGRGDACKD--DFDHDSVPDID
COOH	926	DICPENVDISETDFRRFQMIPLDPKGTSQNDPNWVVRHQGKELVQTVNCDPGLAVGYDEF
	986	NAVDFSGTFFINTERDDDYAGFVFGYQSSSRFYVVMWKQVTQSYWDTNPTRAQGYSGLSV
	1046	KVVNSTTGPGEHLRNALWHTGNTPGQVRTLWHDPRHIGWKDFTAYRWRLSHRPKTGFIRV
	1106	VMYEGKKIMADSGPIYDKTYAGGRLGLFVFSQEMVFFSDLKYECRDP

FIG. 1

human thrombospondin-2

NH_2	1	MVWRLVLLALWVWPSTQAGHQDKDTTFDLFSISNINRKTIGAKQFRGPDPGVPAYRFVRF
	61	DYIPPVNADDLSKITKIMRQKEGFFLTAQLKQDGKSRGTLLALEGPGLSQRQFEIVSNGP
	121	ADTLDLTYWIDGTRHVVSLEDVGLADSQWKNVTVQVAGETYSLHVGCDLIGPVALDEPFY
	181	EHLQAEKSRMYVAKGSARESHFRGLLQNVHLVFENSVEDILSKKGCQQGQGAEINAISEN
	241	TETLRLGPHVTTEYVGPSSERRPEVCERSCEELGNM
P	277	VQELSGLHVLVNQLSENLKRVSNDNQFLWELIGGPPKTRNMSACWQDGRFFAENE
	332	TWVVDSCTTCTCKKFKTICHQITCPPATCASPSFVEGECCPSCLHSVDG
type 1	381	EEGWSPWAEWTQCSVTCGSGTQQRGRSCDVTSNT-----CLGPSIQTRACSLSKCDTRIRQ
	437	DGGWSHWSPWSSCSVTCGVGNITRIRLCNSPVPQMGGKNCKGSGRETKACQGAPCPI
	494	DGRWSPWSPWSACTVTCAGGIRERTRVCNSPEPQYGGKACVGDVQERQMCNKRSCPV
type 2	551	DGCLSNP--CFPGA---QCS---SFPDGSWSCGFCPVGFLG------------NGTHCEDL
	592	DECALVPDICFSTSKVPRCVN----TQPGFHCLPCPPRYRGNQPVGVGLEAAKTEKQVCEPE
	650	NPCKDKTHNCHKHA---ECIYLGHFSDPMYKCE-CQTGYAG-------------DGLICGE
	694	DSDLDGWPNLNLVCATNATYHCIK
type 3	718	DNCPHLPNSGQEDFDKDGLGDACDD--DDDNDGVTDEK
	748	DNCQLLFNPRQADYDKDEVGDRC
	777	DNCPYVHNPAQIDTDNNGEGDACSV--DIDGDDVFNER
	813	DNCPYVYNTDQRDTDGDGVGDHC
	836	DNCPLVHNPDQTDVDNDLVGDQCDNNEDIDDDGHQNNQ
	874	DNCPYISNANQADHDRDGQGDACDP--DDDNDGVPDDR
	910	DNCRLVFNPDQEDLDGDGRGDICKD--DFDNDNIPDID
COOH	946	DVCPENNAISETDFRNFQMVPLDPKGTTQIDPNWVIRHQGKELVQTANSDPGIAVGFDEF
	1006	GSVDFSGTFYVNTDRDDDYAGFVFGYQSSSRFYVVMWKQVTQTYWEDQPTRAYGYSGVSL
	1066	KVVNSTTGTGEHLRNALWHTGNTPGQVRTLWHDPRNIGWKDYTAYRWHLTHRPKTGYIRV
	1126	LVHEGKQVMADSGPIYDQTYAGGRLGLFVFSQEMVYFSDLKYECRDI

FIG. 2

human COMP

```
         1  MVPDTACVLLLTLAALGASGQGQSPLGSDLGPQMLRELQETNAALQDVRDWLRQQVREIT

        61  FLKNTVMECDACGMQQSVRTGLPSVRPL

        89  LHCAPGF---CFPGVACIQTESGGR-CGPCPAGFTG-------------NGSHCTDV
       129  NECNAHP---CFPRVRCINTSPGFR-CEACPPGYSGPTHQGVGLAFAKANKQVCTDI
Type 2 182  NECETGQHN-CVPNSVCINTRGSFQ-CGPCQPGFVG------------DQASGCQRGAQ
       227  RFCPDGSPSECHEHADCVLERDGSRSCV-CRVGWAG-------------NGILCGR

       269  DTDLDGFPDEKLRCPEPQCRK

       290  DNCVTVPNSGQEDVDRDGIGDACD--PDADGDGVPNEK
       326  DNCPLVRNPDQRNTDEDKWGDAC
       349  DNCRSQKNDDQKDTDQDGRGDACD--DDIDGDRIRNQA
Type 3 385  DNCPRVPNSDQKDSDGDGIGDAC
       408  DNCPQKSNPDQADVDHDFVGDACDSDQDQDGDGHQDSR
       446  DNCPTVPNSAQEDSDHDGQGDACD--DDDDNDGVPDSR
       482  DNCRLVPNPGQEDADRDGVGDVCQ--DDFDADKVVDKI

       518  DVCPENAEVTLTDFRAFQTVVLDPEGDAQIDPNWVVLNQGREIVQTMNSDPGLAVGYTAF
COOH   578  NGVDFEGTFHVNTVTDDDYAGFIFGYQDSSSFYVVMWKQMEQTYWQANPFRAVAEPGIQL
       638  KAVKSSTGPGEQLRNALWHTGDTESQVRLLWKDPRNVGWKDKKSYRWFLQHRPQVGYIRV
       698  RFYEGPELVADSNVVLDTTMRGGRLGVFCFSQENIIWANLRYRCNDTIPEDYETHQLRQA
```

FIG. 3

```
CAGCACCCAG CTCCCCGCCA CCGCC ATG GTC CCC GAC ACC GCC TGC GTT CTT      52
                            Met Val Pro Asp Thr Ala Cys Val Leu
                             1               5

CTG CTC ACC CTG GCT GCC CTC GGC GCG TCC GGA CAG GGC CAG AGC CCG     100
Leu Leu Thr Leu Ala Ala Leu Gly Ala Ser Gly Gln Gly Gln Ser Pro
 10                  15                  20                  25

TTG GGC TCA GAC CTG GGC CCG CAG ATG CTT CGG GAA CTG CAG GAA ACC     148
Leu Gly Ser Asp Leu Gly Pro Gln Met Leu Arg Glu Leu Gln Glu Thr
                 30                  35                  40

AAC GCG GCG CTG CAG GAC GTG CGG GAC TGG CTG CGG CAG CAG GTC AGG     196
Asn Ala Ala Leu Gln Asp Val Arg Asp Trp Leu Arg Gln Gln Val Arg
             45                  50                  55

GAG ATC ACG TTC CTG AAA AAC ACG GTG ATG GAG TGT GAC GCG TGC GGG     244
Glu Ile Thr Phe Leu Lys Asn Thr Val Met Glu Cys Asp Ala Cys Gly
         60                  65                  70

ATG CAG CAG TCA GTA CGC ACC GGC CTA CCC AGC GTG CGG CCC CTG CTC     292
Met Gln Gln Ser Val Arg Thr Gly Leu Pro Ser Val Arg Pro Leu Leu
     75                  80                  85

CAC TGC GCG CCC GGC TTC TGC TTC CCC GGC GTG GCC TGC ATC CAG ACG     340
His Cys Ala Pro Gly Phe Cys Phe Pro Gly Val Ala Cys Ile Gln Thr
 90                  95                 100                 105

GAG AGC GGC GGC CGC TGC GGC CCC TGC CCC GCG GGC TTC ACG GGC AAC     388
Glu Ser Gly Gly Arg Cys Gly Pro Cys Pro Ala Gly Phe Thr Gly Asn
            110                 115                 120
```

FIG. 4A

```
GGC TCG CAC TGC ACC GAC GTC GAT GGT GGC TGG AGC CAC TGG TCC CCG        436
Gly Ser His Cys Thr Asp Val Asp Gly Gly Trp Ser His Trp Ser Pro
            125                     130                     135

TGG TCA TCT TGT TCT GTG ACA TGT GGT GAT GGT GTG ATC ACA AGG ATC        484
Trp Ser Ser Cys Ser Val Thr Cys Gly Asp Gly Val Ile Thr Arg Ile
        140                     145                     150

CGG CTC TGC AAC TCT CCC AGC CCC CAG ATG AAC GGG AAA CCC TGT GAA        532
Arg Leu Cys Asn Ser Pro Ser Pro Gln Met Asn Gly Lys Pro Cys Glu
    155                     160                     165

GGC GAA GCG CGG GAG ACC AAA GCC TGC AAG AAA GAC GCC TGC CCC ATC        580
Gly Glu Ala Arg Glu Thr Lys Ala Cys Lys Lys Asp Ala Cys Pro Ile
170                 175                     180                 185

AAT GGA GGC TGG GGT CCT TGG TCA CCA TGG GAC ATC TGT TCT GTC ACC        628
Asn Gly Gly Trp Gly Pro Trp Ser Pro Trp Asp Ile Cys Ser Val Thr
                190                     195                     200

TGT GGA GGA GGG GTA CAG AAA CGT AGT CGT CTC TGC AAC AAC CCC ACA        676
Cys Gly Gly Gly Val Gln Lys Arg Ser Arg Leu Cys Asn Asn Pro Thr
            205                     210                     215

CCC CAG TTT GGA GGC AAG GAC TGC GTT GGT GAT GTA ACA GAA AAC CAG        724
Pro Gln Phe Gly Gly Lys Asp Cys Val Gly Asp Val Thr Glu Asn Gln
        220                     225                     230

ATC TGC AAC AAG CAG GAC TGT CCA ATC TAG  A                             755
Ile Cys Asn Lys Gln Asp Cys Pro Ile  *
    235                     240
```

FIG. 4B

```
CAGCACCCAG CTCCCCGCCA CCGCC ATG GTC CCC GAC ACC GCC TGC GTT CTT     52
                            Met Val Pro Asp Thr Ala Cys Val Leu
                             1               5

CTG CTC ACC CTG GCT GCC CTC GGC GCG TCC GGA CAG GGC CAG AGC CCG    100
Leu Leu Thr Leu Ala Ala Leu Gly Ala Ser Gly Gln Gly Gln Ser Pro
 10                  15                  20                  25

TTG GGC TCA GAC CTG GGC CCG CAG ATG CTT CGG GAA CTG CAG GAA ACC    148
Leu Gly Ser Asp Leu Gly Pro Gln Met Leu Arg Glu Leu Gln Glu Thr
                 30                  35                  40

AAC GCG GCG CTG CAG GAC GTG CGG GAC TGG CTG CGG CAG CAG GTC AGG    196
Asn Ala Ala Leu Gln Asp Val Arg Asp Trp Leu Arg Gln Gln Val Arg
             45                  50                  55

GAG ATC ACG TTC CTG AAA AAC ACG GTG ATG GAG TGT GAC GCG TGC GGG    244
Glu Ile Thr Phe Leu Lys Asn Thr Val Met Glu Cys Asp Ala Cys Gly
         60                  65                  70

ATG CAG CAG TCA GTA CGC ACC GGC CTA CCC AGC GTG CGG CCC CTG CTC    292
Met Gln Gln Ser Val Arg Thr Gly Leu Pro Ser Val Arg Pro Leu Leu
     75                  80                  85

CAC TGC GCG CCC GGC TTC TGC TTC CCC GGC GTG GCC TGC ATC CAG ACG    340
His Cys Ala Pro Gly Phe Cys Phe Pro Gly Val Ala Cys Ile Gln Thr
 90                  95                 100                 105

GAG AGC GGC GGC CGC TGC GGC CCC TGC CCC GCG GGC TTC ACG GGC AAC    388
Glu Ser Gly Gly Arg Cys Gly Pro Cys Pro Ala Gly Phe Thr Gly Asn
                110                 115                 120

GGC TCG CAC TGC ACC GAC GTC GAG GAG GGC TGG TCT CCG TGG GCA GAG    436
Gly Ser His Cys Thr Asp Val Glu Glu Gly Trp Ser Pro Trp Ala Glu
            125                 130                 135

TGG ACC CAG TGC TCC GTG ACG TGT GGC TCT GGG ACC CAG CAG AGA GGC    484
Trp Thr Gln Cys Ser Val Thr Cys Gly Ser Gly Thr Gln Gln Arg Gly
        140                 145                 150
```

FIG. 5A

```
CGG TCC TGT GAC GTC ACC AGC AAC ACC TGC TTG GGG CCC TCG ATC CAG      532
Arg Ser Cys Asp Val Thr Ser Asn Thr Cys Leu Gly Pro Ser Ile Gln
    155                 160                 165

ACA CGG GCT TGC AGT CTG AGC AAG TGT GAC ACC CGC ATC CGG CAG GAC      580
Thr Arg Ala Cys Ser Leu Ser Lys Cys Asp Thr Arg Ile Arg Gln Asp
170                 175                 180                 185

GGC GGC TGG AGC CAC TGG TCA CCT TGG TCT TCA TGC TCT GTG ACC TGT      628
Gly Gly Trp Ser His Trp Ser Pro Trp Ser Ser Cys Ser Val Thr Cys
                190                 195                 200

GGA GTT GGC AAT ATC ACA CGC ATC CGT CTC TGC AAC TCC CCA GTG CCC      676
Gly Val Gly Asn Ile Thr Arg Ile Arg Leu Cys Asn Ser Pro Val Pro
            205                 210                 215

CAG ATG GGG GGC AAG AAT TGC AAA GGG AGT GGC CGG GAG ACC AAA GCC      724
Gln Met Gly Gly Lys Asn Cys Lys Gly Ser Gly Arg Glu Thr Lys Ala
        220                 225                 230

TGC CAG GGC GCC CCA TGC CCA ATC GAT GGC CGC TGG AGC CCC TGG TCC      772
Cys Gln Gly Ala Pro Cys Pro Ile Asp Gly Arg Trp Ser Pro Trp Ser
    235                 240                 245

CCG TGG TCG GCC TGC ACT GTC ACC TGT GCC GGT GGG ATC CGG GAG CGC      820
Pro Trp Ser Ala Cys Thr Val Thr Cys Ala Gly Gly Ile Arg Glu Arg
250                 255                 260                 265

ACC CGG GTC TGC AAC AGC CCT GAG CCT CAG TAC GGA GGG AAG GCC TGC      868
Thr Arg Val Cys Asn Ser Pro Glu Pro Gln Tyr Gly Gly Lys Ala Cys
                270                 275                 280

GTG GGG GAT GTG CAG GAG CGT CAG ATG TGC AAC AAG AGG AGC TGC CCC      916
Val Gly Asp Val Gln Glu Arg Gln Met Cys Asn Lys Arg Ser Cys Pro
            285                 290                 295

GTG TCT AGA                                                          925
Val Ser Arg
```

FIG. 5B

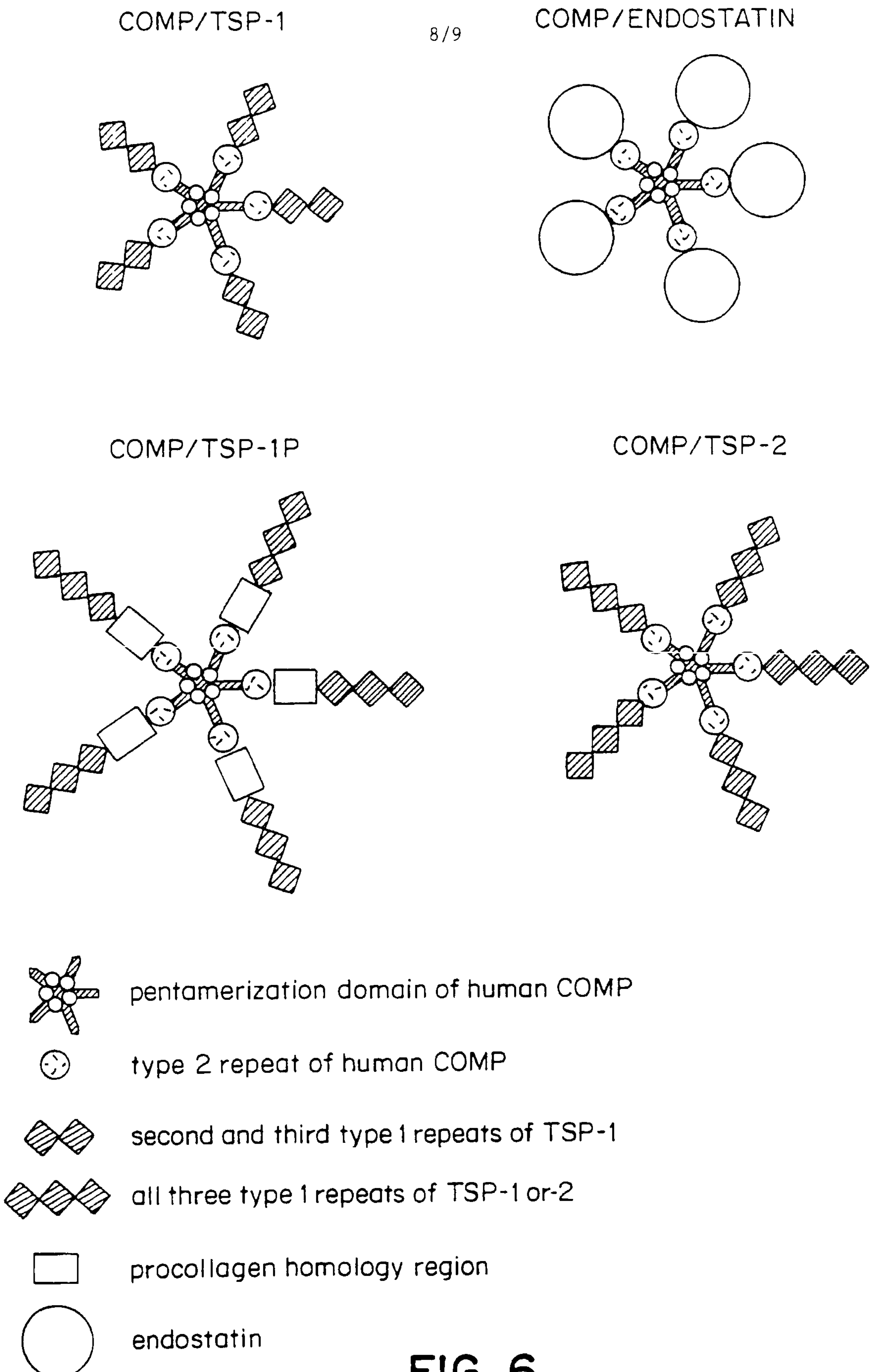
COMP/TSP-1
COMP/ENDOSTATIN
COMP/TSP-1P
COMP/TSP-2
pentamerization domain of human COMP
type 2 repeat of human COMP
second and third type 1 repeats of TSP-1
all three type 1 repeats of TSP-1 or-2
procollagen homology region
endostatin

FIG. 6

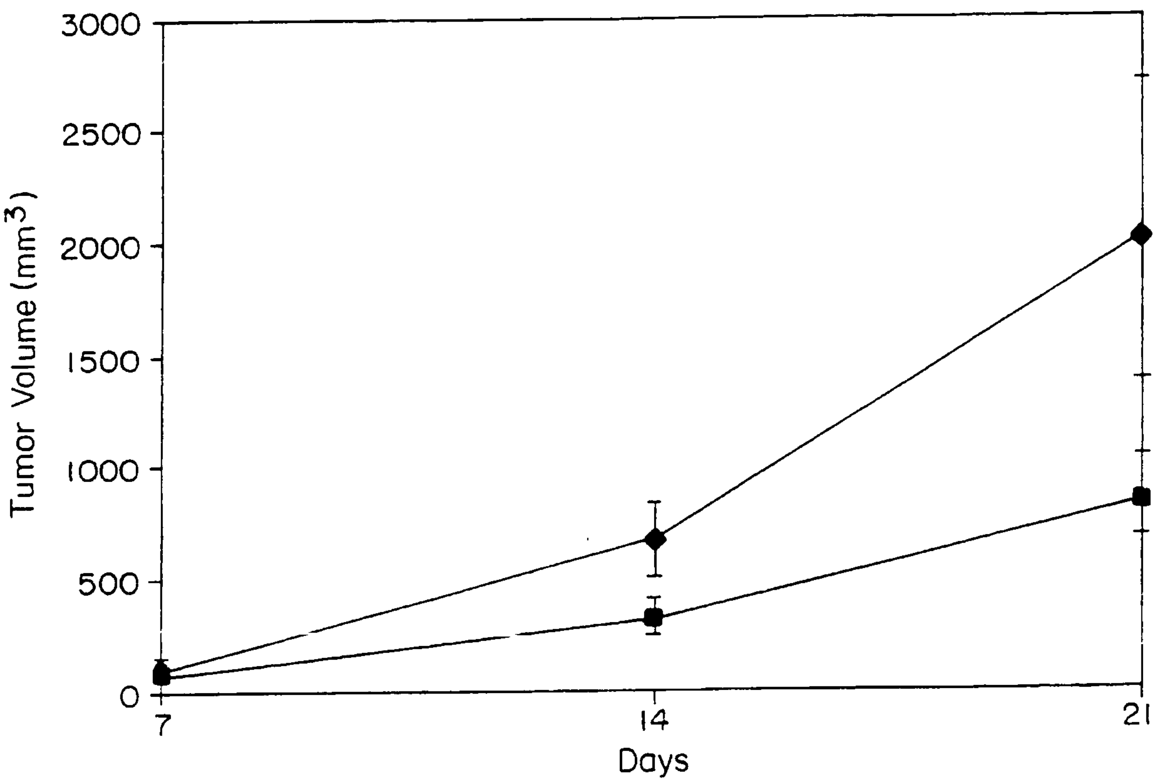

FIG. 7

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02482

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 C12N15/52 C07K14/78 C07K19/00 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, CHEM ABS Data, BIOSIS, MEDLINE, SCISEARCH, BIOTECHNOLOGY ABS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PANETTI TS ET AL: "Interaction of recombinant procollagen and properdin modules of thrombospondin-1 with heparin and fibrinogen/fibrin." JOURNAL OF BIOLOGICAL CHEMISTRY, JAN 1 1999, 274 (1) P430-7, XP002140107 UNITED STATES	1,12,13, 25
A	abstract; figure 2B	2-5,10, 11, 14-24, 26-28
	--- -/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 September 2000	18.10.00
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer Gurdjian, D

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INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 00/02482

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	QABAR AZIZ N ET AL: "A chimeric murine TSP3/human TSP1 is a pentamer with abolished antiangiogenic activity." ANNUAL MEETING OF THE PROFESSIONAL RESEARCH SCIENTISTS ON EXPERIMENTAL BIOLOGY 97;NEW ORLEANS, LOUISIANA, USA; APRIL 6-9, 1997, vol. 11, no. 3, 1997, page A63 XP000914726 FASEB Journal 1997 ISSN: 0892-6638 the whole document ---	1,12,13, 25
A	WO 98 18943 A (CIBA GEIGY AG ;KAJAVA ANDREY (CH); UNIV LAUSANNE (CH); CRAMERI RET) 7 May 1998 (1998-05-07) abstract; claims 1-32 ---	1-5, 10-28,33
A	WO 98 39418 A (ARIAD GENE THERAPEUTICS INC ;GILMAN MICHAEL Z (US)) 11 September 1998 (1998-09-11) claims 1-5 ---	1-5, 10-28,33
A	WO 93 16716 A (UNIV NORTHWESTERN) 2 September 1993 (1993-09-02) claims 1-11; figures SEQ.ID.1,2 ---	1-5, 10-28,33
A	KUNO ET AL: "Molecular cloning of a gene encoding a new type of metalloproteinase-disintegrin family protein with thrombospondin motifs as an inflammation associated gene" JOURNAL OF BIOLOGICAL CHEMISTRY,US,AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS, BALTIMORE, MD, vol. 272, no. 1, 3 January 1997 (1997-01-03), pages 556-562, XP002076038 ISSN: 0021-9258 abstract; figures 5,6 ---	1-5, 10-28,33
Y	WO 96 37621 A (HOESS ADOLF ;PACK PETER (DE); MORPHOSYS PROTEINOPTIMIERUNG (DE)) 28 November 1996 (1996-11-28) claims 1,4-7 ---	6-9, 29-32
Y	EP 0 407 122 A (REPLIGEN CORP) 9 January 1991 (1991-01-09) claims 1-23 ---	9,32
Y	WO 98 54217 A (CHILDRENS MEDICAL CENTER) 3 December 1998 (1998-12-03) abstract; claims 1-15 ---	7,30

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Interr. ıal Application No
PCT/US 00/02482

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with inc. ation,where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 98 51323 A (STRUMAN INGRID ;MARTIAL JOSEPH A (BE); TAYLOR ROBERT (US); UNIV CA) 19 November 1998 (1998-11-19) abstract; claims 1-26 ---	8,31
Y	NGUYEN JOSEPHINE T ET AL: "Adeno-associated virus-mediated delivery of antiangiogenic factors as an antitumor strategy." CANCER RESEARCH, vol. 58, no. 24, 15 December 1998 (1998-12-15), pages 5673-5677, XP000857408 ISSN: 0008-5472 the whole document ---	6,29
A	TOMSCHY ANDREA ET AL: "Homophilic adhesion of E-cadherin occurs by a co-operative two-step interaction of N-terminal domains." EMBO (EUROPEAN MOLECULAR BIOLOGY ORGANIZATION) JOURNAL, vol. 15, no. 14, 1996, pages 3507-3514, XP002147181 ISSN: 0261-4189 the whole document ---	6-9, 29-32
A	TERSKIKH ALEXEY V ET AL: ""Peptabody": A new type of high avidity binding protein." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES, vol. 94, no. 5, 1997, pages 1663-1668, XP002147182 1997 ISSN: 0027-8424 the whole document ---	6-9, 29-32
A	NEWTON GAIL ET AL: "Characterization of human and mouse cartilage oligomeric matrix protein." GENOMICS, vol. 24, no. 3, 1994, pages 435-439, XP002147953 ISSN: 0888-7543 the whole document ---	34-50
A	O'ROURKE KM ET AL: "Thrombospondin 1 and thrombospondin 2 are expressed as both homo- and heterotrimers." JOURNAL OF BIOLOGICAL CHEMISTRY, DEC 15 1992, 267 (35) P24921-4, XP002147183 UNITED STATES the whole document -----	34-50

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 00/02482

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

   Although claims 51-53 are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. [X] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[X] No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

This International Searching Authority found multiple (groups of) inventions in this international application, as follows:

1. Claims: 51-53 partially and 1-5,10-28 ,33

   TSP-1 chimeric protein constructs, corresponding nucleic acids ,vectors ,host cells , method of producing chimeric proteins

2. Claims: 51-53 partially and 34-50

   TSP-2 chimeric protein constructs, corresponding nucleic acids ,vectors ,host cells ,method of producing chimeric proteins

3. Claims: 51-53 partially and 6-9, 29-32

   COMP chimeric protein constructs, not provided in the first and second invention of present application , corresponding nucleic acids ,vectors ,host cells , method of producing chimeric proteins

INTERNATIONAL SEARCH REPORT

Information on patent family members

Intern ..al Application No
PCT/US 00/02482

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 9818943	A	07-05-1998	AU	6909298	A	22-05-1998
			EP	0938571	A	01-09-1999
WO 9839418	A	11-09-1998	AU	6692298	A	22-09-1998
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			JP	11508126	T	21-07-1999
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			ES	2092493	T	01-12-1996
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			AU	7704998	A	30-12-1998
			EP	0996632	A	03-05-2000
WO 9851323	A	19-11-1998	AU	7383298	A	08-12-1998